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Study of the effect of cadmium on the early life stages of brown trout (*Salmo trutta*) at different levels of water hardness

Norwegian Institute for Water Research
– an institute in the Environmental Research Alliance of Norway

REPORT

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Abstract

The effect of water hardness on the chronic toxicity of cadmium to early life stages of brown trout (*Salmo trutta*) has been studied in accordance with OECD Guideline 210; Fish Early Life Stage Test. High-hardness waters were prepared by adding calcium to natural soft water achieving a nominal hardness level of 40 mg $CaCO_3$/l. The experiment consisted of a factorial design comprising three levels of $CaCO_3$ (2.7, 12.8 and 42.7 mg/l) and six levels of cadmium (0, 0.1, 0.32, 1, 3.2, 10 µg/l), including a control group of the natural background water. Eggs of brown trout were fertilised in their respective exposure water and exposed for approximately 120 days in flow-through chambers. In addition to cumulated mortality, hatching success, time-to-hatching and length and weight of larvae at four sampling points (newly fertilised eggs, eyeing stage, hatching and start feeding stage) were observed. Furthermore, body concentrations of calcium and cadmium were measured at the same four stages. The body cadmium concentrations changed a lot over the ontogeny, starting very high and reducing over time, but the body cadmium concentration always remained at least 10 times higher than the control group, and it was always highest in the low-calcium water concentration treatment. The biological effects were not very pronounced for the life stages under study. Even though it was documented significant interaction effects between calcium and cadmium concentrations on cumulated mortality ($p<0.0001$), the mortality in general was very low (0.5–12%), and the high-concentration cadmium treatments were not always producing a higher mortality rate than the control. The clearest effects measured were for size- and weight at start feeding. Clearly, the hatching-to-start-feeding growth rate was highly affected by high cadmium dosages, and mostly so at low $CaCO_3$ concentrations. There was also found a significant ($p < 0.0001$) additive effect of cadmium on hatching trajectories where high levels of cadmium gave a delayed hatching probability. This effect involved a delayed hatching time of less than 10 degreedays. The results are summarised in a NOEC/LOEC table that concludes that LOEC for size and weight at start feeding is 0.95, 3.2 and 3.2 µg/l of water cadmium at water $CaCO_3$ concentrations of 2.7, 12.8 and 42.7 mg/l, respectively. The estimated hardness slope of 0.42 would require extremely low $CaCO_3$ concentrations in order to surpass the prevailing PNEC value (0.08 Cd µg/l). Estimated EC10 values for weight at start-feeding (95% CI) were 0.34 (0.25,0.47), 0.92 (0.38,2.22) and 6.16 (1.92,19.8) µg/l at the same $CaCO_3$ concentrations, yielding a water hardness slope of 1.03. For cumulated mortality and hatching trajectory LOEC is set at 10 µg/l of cadmium for all water hardness levels involved in this study. The results are discussed in light of previous findings confirming that the most profound effects of cadmium on fish relate to post-hatching life stages.

4 keywords, Norwegian	4 keywords, English
1. Kadmium	1. Cadmium
2. Fisk	2. Fish
3. Toksisitet	3. Toxicity
4. Hardhet	4. Water hardness

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Study of the effect of cadmium on the early life stages of brown trout (*Salmo trutta*) at different levels of water hardness

Preface

This report is funded by the International Cadmium Association and has as prime objective to establish key metrics related to the eventual toxic effect of solved cadmium on early life stages of fish, *i.e.*, brown trout (*Salmo trutta*) over a range of water hardness levels, with special emphasis on soft water conditions. The tests were carried out at Syrtveit settefiskanlegg (Syrtveit fish farm), Aust-Agder, Norway, and the staff is acknowledge for their skilful management of the experiments. In fact, the staff at the fish farm conducted most of the daily routines during the experiments. The staff at NIVA performed the sampling, measurements and analysis tasks, as well as wrote the report. This is the final report. Preliminary reports along with key data have been provided the client during July and August this year.

We hope that the report will provide useful information that can provide further insights into the interaction effects of cadmium and water hardness on aquatic organisms.

Oslo, 5 September 2007

Thrond O Haugen

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Summary

The effect of water hardness on the chronic toxicity of cadmium to early life stages of brown trout has been studied in accordance with OECD Guideline 210; Fish Early Life Stage Test. High-hardness waters were prepared by adding calcium to natural soft water achieving a nominal hardness level of 40 mg $CaCO_3$/l. The experiment consisted of a factorial design comprising three levels of $CaCO_3$ (2.7, 12.8 and 42.7 mg/l) and six levels of cadmium (0, 0.1, 0.32, 1, 3.2, 10 µg/l), including a control group of the natural background water. Eggs of brown trout were fertilised in their respective exposure water and exposed for approximately 120 days in flow-through chambers. In addition to cumulated mortality, hatching success, time-to-hatching and length and weight of larvae at four sampling points (newly fertilised eggs, eyeing stage, hatching and start feeding stage) were observed. Furthermore, body concentrations of calcium and cadmium were measured at the same four stages. The body cadmium concentrations changed a lot over the ontogenetic stages, starting very high and reducing over time, but the body cadmium concentration always remained at least 10 times higher than the control group, and it was always highest in the low-calcium water concentration treatment. The biological effects were not very pronounced for the life stages under study. Even though it was documented significant interaction effects between calcium and cadmium concentrations on cumulated mortality ($p<0.0001$), the mortality in general was very low (0.5–12%), and the high-concentration cadmium treatments were not always producing a higher mortality rate than the control. The clearest effects measured were for size- and weight at start feeding. Clearly, the hatching-to-start-feeding growth rate was highly affected by high cadmium dosages, and mostly so at low $CaCO_3$ concentrations. There was also found a significant ($p < 0.0001$) additive effect of cadmium on hatching trajectories where high levels of cadmium gave a delayed hatching probability. This effect involved a delayed hatching time of less than 10 degreedays. The results are summarised in a NOEC/LOEC table that concludes that LOEC for size and weight at start feeding is 0.95, 3.2 and 3.2 µg/l of water cadmium at water $CaCO_3$ concentrations of 2.7, 12.8 and 42.7 mg/l, respectively. The estimated hardness slope of 0.42 would require extremely low $CaCO_3$ concentrations in order to surpass the prevailing PNEC value (0.08 Cd µg/l). Estimated EC10 values for weight at start-feeding (95% CI) were 0.34 (0.25,0.47), 0.92 (0.38,2.22) and 6.16 (1.92,19.8) µg/l at the same $CaCO_3$ concentrations, yielding a water hardness slope of 1.03. For cumulated mortality and hatching trajectory LOEC is set at 10 µg/l of cadmium for all water hardness levels involved in this study. The results are discussed in light of previous findings confirming that the most profound effects of cadmium on fish relate to post-hatching life stages.

1. Background

The December 2005 draft for risk assessment of Cadmium oxide and Cadmium metal (CAS-No.: 1306-19-0 and CAS-No.: 7440-43-9) concludes: "The PNEC for soft water may not be protective for very soft waters" and "There is a need for better information regards the toxic effects of Cd to aquatic organisms under low water hardness conditions." Based on these conclusions and following discussions, the Norwegian Institute for Water Research (NIVA) was asked to propose a project to provide information on the modifying effect of hardness on the chronic toxicity of Cd to fish. The obtained information will be used to propose a regional $PNEC_{soft\ water}$ for the Nordic countries.

It is realized that that water characteristics affect Cd toxicity. Toxicity generally increases with reducing hardness (Hollis et al. 2000; Richards and Playle 1999), reducing concentrations of dissolved organic matter an increasing pH. The existing data on toxicity are, however too scarce to allow a quantitative analysis of the effects of pH and organic matter. For hardness, a correction of chronic values has been proposed, based on the slopes in plots of natural logarithm of chronic values against water hardness for *Daphnia magna* and two species of fish (US-EPA 2001). This hardness correction is recommended for the calculation of $PNEC_{regional}$ for regions with hardness levels in the range 40-200 mg $CaCO_3$/l. In the preliminary Risk Assessment Report the PNEC for water with hardness 40 mg/l has been calculated to 0.08 µg Cd/l, and it has been concluded that down to water hardness of 7-10 mg/l there is no indication of Cd toxicity below 0.08 µg/l. However, data are lacking for effects of Cd in very soft waters (hardness below about 10 mg $CaCO_3$/l) and it is not known if these waters are protected by the proposed PNEC for soft water (0.08 µg/l). It has therefore been suggested to perform further testing to assess the risks of Cd in very soft waters.

A large proportion of lakes in the Nordic countries, and Norway in particular, have hardness levels below 10. To allow risk assessment of Cd in this region it is therefore necessary to extend the hardness correction below harness level of 10 mg $CaCO_3$/l. The present study was therefore initiated to generate information on the effect of water hardness on Cd toxicity to the salmonid fish brown trout (*Salmo trutta*).

2. Material and Methods

2.1 Watershed and background water quality

Based on previous work to obtain NOECs for soft waters for zinc (Zn) (Källqvist et al. 2003), the test was carried out in accordance with OECD Guideline 210, Fish Early Life Stage Test, (OECD 1992), with the modification that eggs are exposed to the test chemical from the time of fertilisation including the swelling phase, as this phase has been shown to be important for effects of other metals (Käinenen et al. 2000). The location of the experiment was chosen based on natural soft water properties, and availability of a previously investigated native fish strain adapted to very soft waters (Dalziel et al. 1995, Källqvist et al. 2003). The experiment was performed at Syrtveit fish farm, Aust-Agder, Norway (**Figure 1**). The fish farm is fed water from lake Byglandsfjord (lake id: 1063). The lake is located within the River Otra (REGINE nr. 0.21) watershed, has a surface area of 33.5 km^2 and is located 203 meters above sea level. River Otra is characterized as a mountain dominated river, having low Ca (0.9 ± 02 mg Ca/L) and low total organic carbon content (2.1+0.7 mg C/l) based on 42 samples from 2000 to 2004. The lower part of the river is slightly affected by acidification. The water quality from Lake Byglandsfjord and up is to a lesser degree affected (Kroglund et al., 2001).

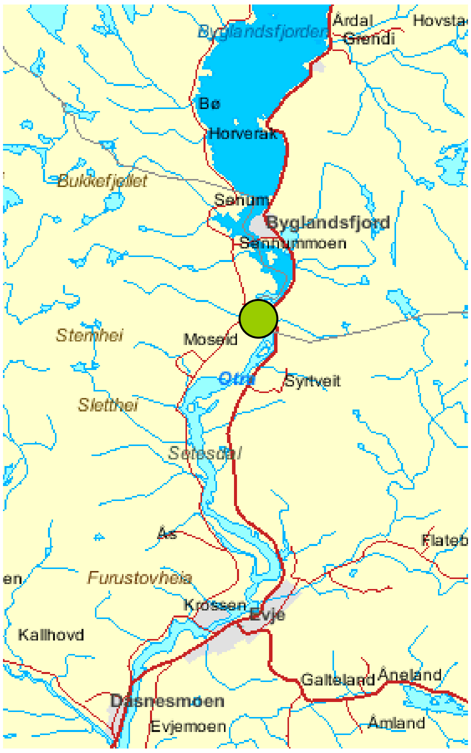


Figure 1. Location of the experimental facilities in the River Otra catchment, southern Norway.

2.2 The test fish

Brown trout (*Salmo trutta*), Lake Byglandsfjord strain, gametes from 1st generation captive parents was obtained from mature individuals. 20 Female fish (length: 52 ± 5 cm) were stripped of their eggs, and the eggs were dry-fertilized with gametes from 14 males (length: 54 ± 2.5 cm). 36 subunits of egg mixtures from all females were added approximately equal amounts of sperm from all males to secure random fertilization (dry fertilization). The eggs were then added their respective experimental water quality (the water activates the sperm). After two minutes the eggs were rinsed (three times) using the experimental water quality and placed in their respective experimental units for swelling in the same water quality. Fertilization rate was determined for the experimental population as a whole at the 2-4 cell stage. Fertilization rate was higher than 98 % in all groups of eggs.

2.3 Test solutions

NaOH treated Lake Byglandsfjord water was added Ca (as $CaCl_2$*$2H_2O$) to obtain 3 Ca-levels. The nominal water hardness levels were 2 (untreated), 10 and 40 mg $CaCO_3$ (see details in Källqvist 2007).

Hardness is caused by multivalent metallic cations. The principal hardness-causing cations are the divalent calcium and magnesium ions. Hardness (in mg/l) as $CaCO_3$ can be calculated as:

$$= M^{2+} \text{ (mg/l) * (100 g/mol } CaCO_3\text{/ atomic weight of } M^{2+)}$$

1 mg Ca/l gives a hardness of 2.5 mg/l, while 1 mg Mg/l gives a hardness of 4.1 mg/l.

2.4 Exposure concentrations

Each water hardness level was added Cd (as $Cd(NO_3)_2$*$4H_2O$) to obtain 5 Cd-levels and a control group. Nominal Cd doses were 0 (untreated), 0,1, 0,3, 1, 3,2, and 10 µg Cd/L.

2.5 Experimental design

Each treatment (18 treatment levels in total, see **Table 1** for labelling practice) was replicated in two separate units randomly placed within the experimental room (**Figure 2**). A minimum of 150 fertilised eggs was placed in each unit at the start of the experiment. Water was supplied to the units at a rate of approximately 30 ml/min by aquarium pumps. This design fulfilled the loading criterion of the Guideline (0,5 g/l/day).

Table 1. An overview of the treatment levels and the labelling practice used in this experiment. Letters indicate the cadmium dosage and the number indicates calcium water concentrations.

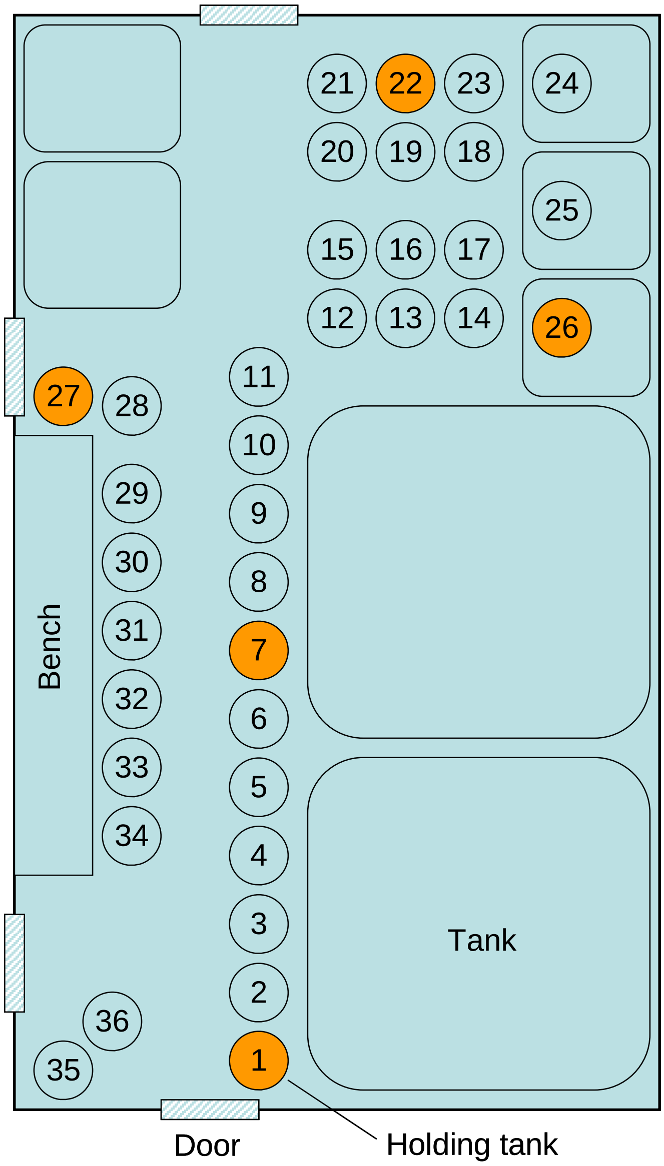
Ca ↓ / Cd →	0	0.1	0.32	1	3.2	10 µg/l
0+	A1	B1	C1	D1	E1	F1
10+	A2	B2	C2	D2	E2	F2
40+	A3	B3	C3	D3	E3	F3

mg $CaCO_3$/l

2.6 Tank set-up

Each exposure set-up consisted of a 90 L black tank filled with 50 L water (**Figure 3**). Water was circulated from the tank to the hatching chamber. Water returned to the 50 L storage by gravity. The stock solution was changed every 14 days, resulting in a total of 13 water exchanges throughout the experimental period. Changing implied pumping out water, addition of new water and chemicals. . Water flow past the eggs was unaffected by this procedure.

The exposure tanks were placed on the floor. A few tanks were placed inside larger rearing tanks due to lack of space. Tanks placed close to the side entrance were influenced by a cold draught. This cold draught influenced the water temperature in some of the groups. In addition to daily temperature measurements, five tanks were equipped with Tiny Tag temperature loggers (Gemini Data Loggers Ltd.; UK). These recorded temperature every ½ hour.



TANK	TREATMENT
1	C2
2	E3
3	E1
4	E3
5	D1
6	C1
7	C2
8	B3
9	F2
10	B2
11	F3
12	E1
13	B2
14	C3
15	F2
16	A2
17	B1
18	A3
19	F1
20	D2
21	A3
22	D2
23	D3
24	D3
25	A1
26	E2
27	A2
28	D1
29	A1
30	B1
31	C1
32	C3
33	F3
34	B3
35	E2
36	F1

Figure 2. Placement of the experimental units in the experimental room. Tanks with additional temperature loggers are indicated in orange. The number of each unit corresponds to tank No. and corresponding treatment level (see **Table 1** for explanation of the treatment codes) is indicated in the table to the right.

The eggs were placed on top of a grid. Upon hatching, the fry would move through the holes and seek cover under the grid. The outlet tubing was covered with mesh to avoid eggs, egg shells and fry escaping the exposure chamber. Each tank was individually numbered and had an additional treatment code. Each tank has its own set of equipment for cleaning etc to avoid and minimize cross contaminations.

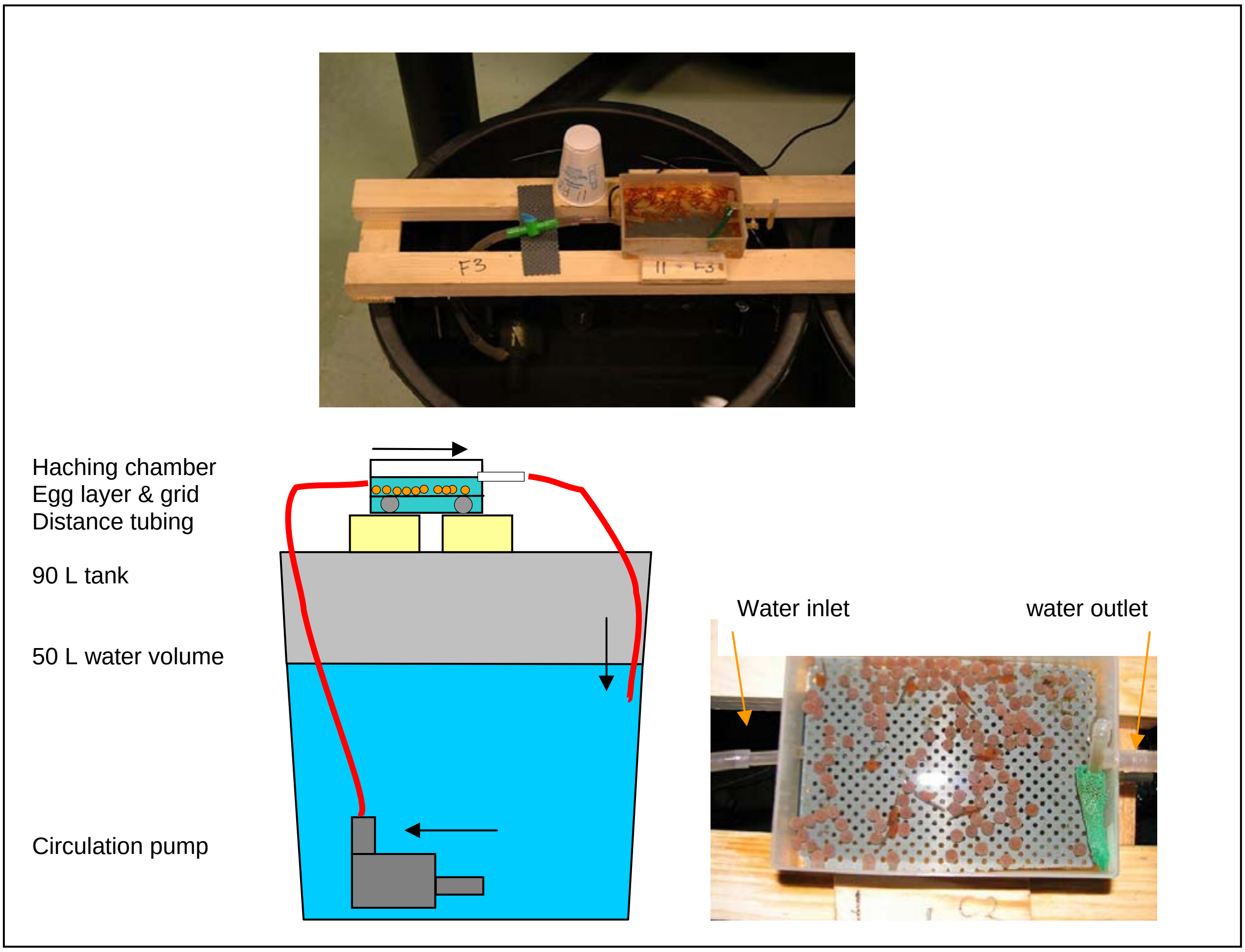


Figure 3. Exposure tank system.

2.7 Sampling protocol

Monitoring of mortality and water flow was conducted daily. Conductivity, temperature and oxygen saturation was measured 3 times a week in all tanks throughout the experiment. Conductivity and temperature was measured using a WTW - Cond 340i/SET, while an OxyGuard HandyPolaris was used for oxygen measurements.

Water samples were obtained from dilution water and all 36 tanks every 14 days throughout the experiment. Dilution water samples were analyzed for main ionic composition (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}), nitrogen compounds (total N and Nitrate), total organic carbon (TOC) and metals (Al, Fe). All experimental groups were analyzed for Cd concentration.

At four times, representing distinct developmental stages throughout the experiment, eggs and fry were sampled according to **Table 2**

Table 2. Sampling stages and variables for biological sampling.

Biological sampling	Stage			
Variables	Fertilization	Eyed eggs	Hatching	Startfeeding
Fertilization	n=50*			
Wet weight	n=10	n=10	n=10	n=10
length/diameter	n=10	n=10	n=10	n=10
Cd concentration	n=10	n=10	n=10	n=10
Ca concentration	n=10	n=10	n=10	n=10

Fertilization on the experimental group as a whole was confirmed at the 2-4 cell stage. All sampling groups were digitally photographed on scaled background, and diameter/length was determined using ImageJ software. Four separate measurements of egg diameter on each individual egg were performed at the two first sampling points to account for shape variation. Length of fry was determined as total length.

After photography, eggs were weighted individually and placed in acid-washed vials for later determination of metal concentration (Ca and Cd, provided as µg/g body tissue wet weight). Metal concentration was determined as grouped values for each tank at each sampling time.

2.8 Chemical analysis

All water and biological samples were analyzed at NIVA's laboratory using accredited methods. NIVA uses an ICPMS instrument, Perkin Elmer Elan 6000, which is a state of the art quadropole mass-spectrometer with plasma source. The ICPMS methods for analysis of metal in water, biota and other environmental samples have been developed to meet normal accreditation quality assurance criteria by the Norwegian Accreditation authority to comply with ISO/EN 17025. The ICPMS methods have been thoroughly tested and use normal QA procedures such as: A careful selected set of internal standards, matrix matching of calibration standards and samples, calibration standards bought from recognized vendors, daily checks with both in house developed QA samples and internationally accepted Certified Reference Materials (CRMs). QA charts are prepared daily to overlook the general performance of the methods, and actions are taken if our QA limits are violated. Normally action limits on CRMs are between 10-20 pct. NIVAs lab have been accredited since 1993 and have now a well performing QA system.

Freshwater samples are preserved with nitric acid on arrival of the laboratory in a class 100 LAF bench in a class 100 000 clean-room laboratory, and left overnight to ensure dissolution of particles and prevent wall adsorption effects.

Typical CRMs are delivered from:

- NRC-INMS (National Research Council Canada - Institute for National Measurement Standards, http://inms-ienm.nrc-cnrc.gc.ca)
- NRC-INMS MESS-3 Marine Sediment Reference Materials
- NRC-INMS HISS-1 Marine Sediment Reference Materials
- NRC-CNRC DORM-2 Dogfish Muscle Certified Reference Material for Trace Metals
- NRC-CNRC DOLT-3 Dogfish Liver Certified Reference Material for Trace Metals
- NIVA in house marine sediment from Bjørvika, Oslo harbour (high metal load)
- NRC- INMS SLRS-4 Riverine Water for trace metals (used for water)

NIVA participates regularly in both national and international inter-calibration of laboratories. Especially NIVA participate 2- 4 times annually in the well recognized QUASIMEME program for environmental pollutants/compounds in marine environmental samples. NIVA's results for metals are normally within the accepted limits (Z-scores) for good quality.

2.9 Statistics

This study involves a two-way factorial design of two focal predictor variables, namely calcium and cadmium concentrations. Hence, in all models fitted this factorial design is used as the basis model. In addition, this study has duplicates of each treatment level, and also some of the response variables (egg diameter and egg diameter at the eye stage) uses replicated measurements on the same individuals (to take measurement error into account). Throughout this study, replicate effects have been modelled as random effects and cadmium and calcium effects as fixed effects. Consequently, most models have been fitted using mixed models generalised linear models (GLM, McCullagh & Nelder 1989) where the link function depends on the nature of the response variable (continuous variables use identity link and binomial variables use logit link). Mixed model GLMs were fitted by using the Restricted Maximum Likelihood method. The measurement error information in the egg diameter traits was included in the analyses as a weighing factor using the inverse of the within-individual measurement variance. In summary, the core model used to fit the response data (y) was:

$$y_{ijk} = \alpha_{ij} + \beta_i x_i + \beta_j x_j + \beta_{ij} x_i x_j + \gamma_{ijk} + \varepsilon_{ijk}$$

where x_i and x_j are the two fixed effects, and α (intercept) and βs correspond to parameters under estimation, and γ_{ijk} is the between replicate variance estimate. ε is the residual variation assumed to be N~(0,1) distributed for a given link function. All models, but the survival analysis, were fitted using the GLM-procedure implemented in R version 2.4.1 (http://www.r-project.org/).

The cumulated mortality trajectories were modelled as Cox proportional hazards regression models using the method described in Andersen & Gill (1982). The assumptions behind proportional hazards were tested using the method described in Grambsch & Therneau (1994). The models were fitted using routines implemented in the SURVIVAL library in R version 2.4.1.

LOEC levels were assessed from ordinary post-hoc contrast tests where each cadmium treatment level within a given calcium level was tested against the control group (A). The lowest cadmium treatment level that had a significant (*i.e.*, $p<0.05$) different response level – in a negative direction – was defined as the LOEC for the particular response trait.

EC10 levels were estimated using ordinary linear regression models where traits that showed consistent negative cadmium-dose responses. In these regression models, the average control response value was defined as 1 yielding other responses as fractions of the control values. These fractions were modelled over averaged measured cadmium concentrations with separate models for each water hardness level. The EC10 values could then be assessed by finding the corresponding x-value at $y = 0.9$ (i.e., 10% drop compared to control). The confidence intervals of EC10 were assessed from the dose-response regression parameters variance-covariance matrix.

3. Results

3.1 Dilution water

Water from Lake Byglandsfjord was used as dilution water. Chemical composition of Lake Byglandsfjord water is presented in **Table 3**. The water hardness based on the measured Ca concentrations during the experiment was of 1.34 ± 0.07 mg $CaCO_3$/l. The water source is slightly affected by acidification. To counteract the negative effects of primarily aluminium at low pH conditions, lye (NaOH) was added to the hatchery water. Based on known stable Na and Cl concentrations and constant Na:Cl ratio in Lake Byglandsfjord (Kroglund et al. 2001), the amount of added Na, as well as conductivity increase was calculated. The addition of base increases pH from values of 6.0±0.1 to values around 6.5. The Na-concentration increased with 0.51±0.10 mg/l from December 2006 to April 2007, while conductivity increased from 1.23±0.07 to 1.35±0.09 mS/m and ANC from 32±2 to 56 ± 7 µeq/l. While the concentration of labile-Al is within limits that are associated with fish damage at pH values of 6.2 and lower, the reported concentrations have no known effects when pH is 6.4 or higher (Kroglund et al. 2007). The elevated values measured are due to analytical limitations, caused by the ion exchangers retaining colloidal-Al, falsely indicating the presence of toxic aluminium. Acid neutralizing capacity (ANC) is a measure of water sensitivity to acidification. The resulting ANC value after treatment was well above the threshold values for biological effect on salmonid populations (Lien et al. 1996, Lydersen et al. 2004), and both pH and LAl levels are well above reported limits for effect during early development in salmonids (Skogheim and Rosseland, 1984).

Table 3. Composition of water from the lake Byglandsfjord after water treatment with NaOH. Labile cationic aluminium (LAl) was calculated by subtracting the Non-labile Al concentration (NLAl) from the Acid-reactive Al fraction (RAl). The ANC-value is calculated as: ∑ base cations minus ∑ strong acid anions (Reuss and Johnson, 1985)

Date	pH	Conductivity mS/m	Alkalinity µmol/l	Ca mg/l	Mg mg/l	Na mg/l	K mg/l	SO4 mg/l	Cl mg/l
07.12.2006	6.48	1.30	68	0.72	0.19	1.32	0.14	1.11	1.24
17.12.2006	6.42	1.31	65	0.81	0.16	1.38	0.14	1.11	1.21
03.01.2007	6.5	1.29	66	0.84	0.16	1.33	0.14	1.2	1.34
23.01.2007	6.53	1.54	66	0.84	0.20	1.6	0.15	1.18	1.76
12.02.2007	6.51	1.34	65	0.8	0.17	1.47	0.13	1.11	1.33
02.03.2007	6.52	1.31	65	0.77	0.16	1.37	0.13	1.05	1.28
26.03.2007	6.71	1.39	73	0.83	0.17	1.62	0.13	1.06	1.32
Mean ± SD	6.52 ± 0.08	1.35 ± 0.09	67 ± 3	0.80 ± 0.04	0.17 ± 0.02	1.44 ± 0.13	0.14 ± 0.01	1.12 ± 0.06	1.35 ± 0.19

Date	Al µg/l	R-Al µg/l	NLAl µg/l	LAl* µg/l	Fe µg/l	Total N µg/l	NO3-N µg/l	Total organic C mg C/l	ANC* µeq/l
07.12.2006	84	38	30	8	32	175	70	1.8	65
17.12.2006	90	43	36	7	22	195	95	1.9	53
03.01.2007	86	68	47	21	34	185	88	2.0	47
23.01.2007	86	44	38	6	29	210	81	2.0	52
12.02.2007	100	55	46	9	42	170	77	2.3	55
02.03.2007	95	56	44	12	37	155	76	2.0	51
26.03.2007	91	51	38	13	37	165	79	1.8	64
Mean ± SD	90 ± 6	51 ± 10	40 ± 6	11 ± 5	33 ± 7	179 ± 19	81 ± 8	1.97 ± 0.17	56 ± 7

The Cd concentration in Lake Byglandsfjord water was measured in all tanks with no added Cd at 6 sampling dates during the experimental period. The Cd level (0.017 ± 0.01µg/l, n=36) in Lake Byglandsfjord water was comparable to the median levels in Norwegian lakes (n=985) reported by Skjelkvaale et al. (1999)

3.2 Water chemistry

The addition of $CaCl_2$ increased conductivity from values of 1.53 ± 0,04 mS/m to 4.27 ± 0.27 mS/m and 12.25 ± 0.38 mS/m for the medium and high hardness groups, respectively (**Table 4**). The resulting calculated Ca concentration was 4.06±0.11 and 16.25±0.38 mg/L, respectively. The background Ca-concentration was 0.80±0.04 mg Ca/l (**Table 3**).

Table 4. Conductivity (mS/m), estimated Ca (mg/l) concentrations based on conductivity measurements and measured Cd (µg/l) concentration in all experimental groups.

Group	Tank no.	Nominal Ca addition (mg/l)	Conductivity mS/m (n= 37-42)	Ca-addition mg/l	Nominal Cd	Measured Cd (µg/l) (n=6)
A1	25	0+	1.50±0.23	0	0	0.015±0.006
A1	29	0+	1.51±0.23	0	0	0.012±0.004
B1	17	0+	1.52±0.23	0	0.1	0.134±0.035
B1	30	0+	1.57±0.31	0	0.1	0.127±0.031
C1	6	0+	1.48±0.20	0	0.3	0.302±0.032
C1	31	0+	1.50±0.22	0	0.3	0.318±0.033
D1	5	0+	1.48±0.23	0	1	0.941±0.074
D1	28	0+	1.57±0.31	0	1	0.960±0.092
E1	3	0+	1.59±0.41	0	3.2	2.910±0.267
E1	12	0+	1.59±0.35	0	3.2	3.064±0.336
F1	19	0+	1.57±0.32	0	10	8.470±2.385
F1	36	0+	1.51±0.44	0	10	8.595±2.159
A2	16	10+	4.22±0.17	4.00±0.20	0	0.017±0.012
A2	27	10+	4.22±0.16	4.00±0.30	0	0.012±0.005
B2	10	10+	4.40±0.31	4.30±0.33	0.1	0.154±0.052
B2	13	10+	4.33±0.21	4.10±0.30	0.1	0.143±0.041
C2	1	10+	4.10±0.15	3.90±0.28	0.3	0.325±0.023
C2	7	10+	4.30±0.19	4.20±0.30	0.3	0.314±0.018
D2	20	10+	4.25±0.20	4.10±0.30	1	0.944±0.084
D2	22	10+	4.27±0.33	4.00±0.40	1	0.951±0.098
E2	26	10+	4.31±0.27	4.00±0.40	3.2	3.134±0.176
E2	35	10+	4.30±0.46	4.00±0.60	3.2	3.028±0.117
F2	9	10+	4.27±0.37	4.00±0.60	10	8.162±2.446
F2	15	10+	4.30±0.38	4.15±0.60	10	8.700±2.322
A3	18	40+	12.36±0.50	16.15±0.70	0	0.020±0.007
A3	21	40+	11.94±0.44	15.50±0.63	0	0.025±0.017
B3	8	40+	12.14±0.43	15.90±0.62	0.1	0.132±0.019
B3	34	40+	12.29±0.35	16.00±0.55	0.1	0.128±0.022
C3	14	40+	12.31±0.38	16.10±0.60	0.3	0.325±0.028
C3	32	40+	12.26±0.32	16.00±0.51	0.3	0.293±0.035
D3	23	40+	12.11±0.35	15.80±0.51	1	0.923±0.034
D3	24	40+	12.40±0.37	16.15±0.56	1	0.920±0.063
E3	2	40+	11.96±0.39	15.50±0.60	3.2	2.996±0.114
E3	4	40+	12.48±0.30	16.20±0.50	3.2	3.112±0.118
F3	11	40+	12.50±0.35	16.20±0.55	10	8.187±2.093
F3	33	40+	12.24±0.41	16.00±0.60	10	8.318±2.224

The measured Cd concentrations were in correspondence with the nominal concentrations, with means ± SD (all treatments within Cd-groups combined. n=36) of A: 0.017 ± 0.010. B: 0.137 ± 0.035. C: 0.0313 ± 0.029. D: 0.940 ± 0.072. E: 3.041 ± 0.203 and F: 9.304 ± 0.757 µg/l.

3.3 Temperature

The temperature in all groups showed some variation during the experiment, mostly due to climatic conditions, causing room temperature to decrease in the middle of the experimental period (**Figure 4**). In addition, changing of water in the 50 L holding tanks caused a transient temperature drop which was compensated for within 12 hours. All over, the mean temperature in the holding tanks varied from 5.7 to 6.5 °C. In particular, tank 1 had a deviating temperature profile (located close to a door). Therefore, all results of statistical tests were checked for sensitivity towards inclusion of data from this tank.

Table 5. Temperatures as mean ± SD for all groups during the experiment, and accumulated degree days at biological sampling points.

Group	Tank No.	Cd-exposure	Hardness	Temperature		Accumulated degree-days at sampling			
		Cd (µg/L)	Ca (mg/L)	Mean	SD	Egg	Eyed egg	Hatch	Start feed
A1	25-A1	0	0+	6,5	1,0	27	216	502	682
A1	29-A1	0	0+	6,4	1,0	27	214	498	674
A2	16-A2	0	10+	6,5	1,0	27	216	504	683
A2	27-A2	0	10+	6,4	1,0	27	214	499	674
A3	18-A3	0	40+	6,5	1,0	27	215	500	679
A3	21-A3	0	40+	6,5	1,0	26	215	503	681
B1	17-B1	0,1	0+	6,5	1,0	27	217	502	682
B1	30-B1	0,1	0+	6,4	1,0	27	211	493	696
B2	10-B2	0,1	10+	6,3	1,1	27	211	484	687
B2	13-B2	0,1	10+	6,5	1,1	27	217	503	681
B3	8-B3	0,1	40+	6,2	1,1	27	207	471	674
B3	34-B3	0,1	40+	6,3	1,0	27	208	484	686
C1	6-C1	0,3	0+	6,1	1,1	27	206	466	668
C1	31-C1	0,3	0+	6,4	1,0	27	213	495	698
C2	1-C2	0,3	10+	5,7	1,1	25	187	428	653
C2	7-C2	0,3	10+	6,2	1,1	27	206	469	671
C3	14-C3	0,3	40+	6,4	1,0	27	215	498	676
C3	32-C3	0,3	40+	6,4	1,0	27	212	496	672
D1	5-D1	1	0+	6,1	1,1	27	205	463	663
D1	28-D1	1	0+	6,5	1,0	27	214	501	678
D2	20-D2	1	10+	6,5	1,0	26	216	507	686
D2	22-D2	1	10+	6,5	1,0	26	215	502	680
D3	23-D3	1	40+	6,4	1,1	27	214	496	675
D3	24-D3	1	40+	6,5	1,0	27	216	504	683
E1	3-E1	3,2	0+	6,0	1,1	26	199	456	655
E1	12-E1	3,2	0+	6,4	1,0	27	215	498	676
E2	26-E2	3,2	10+	6,5	1,1	27	215	499	678
E2	35-E2	3,2	10+	6,1	1,0	26	201	464	663
E3	2-E3	3,2	40+	6,0	1,1	26	198	453	650
E3	4-E3	3,2	40+	6,1	1,1	27	203	461	660
F1	19-F1	10	0+	6,5	1,0	27	216	505	685
F1	36-F1	10	0+	6,0	1,1	27	196	455	651
F2	9-F2	10	10+	6,3	1,1	27	209	478	681
F2	15-F2	10	10+	6,5	1,0	27	215	503	683
F3	11-F3	10	40+	6,3	1,1	27	214	488	690
F3	33-F3	10	40+	6,4	1,0	27	211	492	697

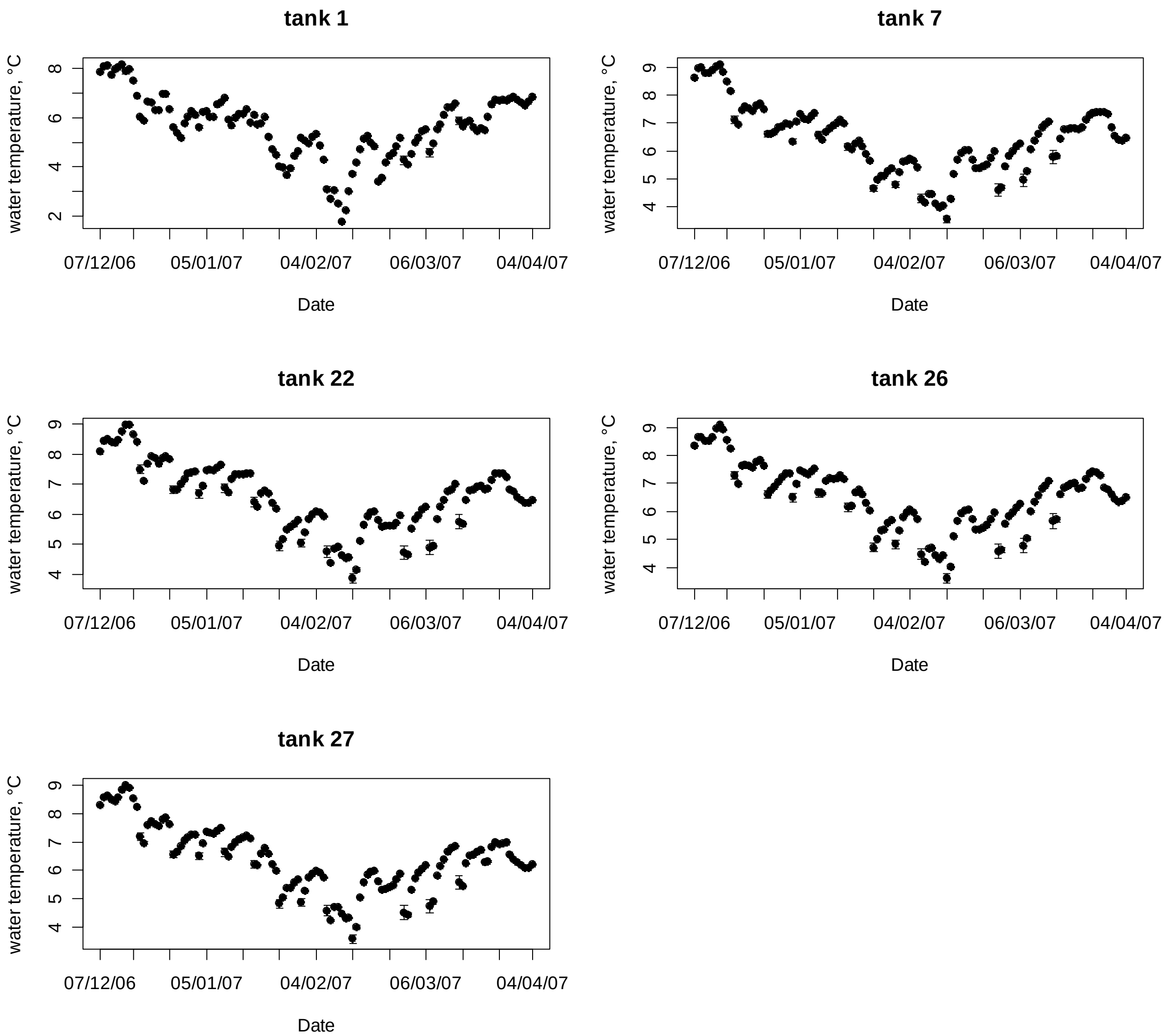


Figure 4. Temperature profiles derived from the five temperature data loggers. Error bars correspond to standard deviations for the daily means.

3.4 Body cadmium and calcium concentrations

The body concentrations of Cd and Ca changed dramatically over the experimental period (**Figure 5**). The egg concentrations followed the water concentrations almost without modifications, and body cadmium concentrations were highest at the lowest water calcium concentration. For the eyed larvae this pattern was even more pronounced. For the start feeding larvae the close correlation between water cadmium and calcium concentrations with body tissue concentrations was modified so as to lower the body concentrations for high-concentration Cd treatment levels. However, the body Cd concentrations remained highest at high cadmium water concentrations and also highest at low Ca water concentrations. In fact, all cadmium treatment levels showed evidence of at least a 5-fold increase in body cadmium concentrations in the start feeding larvae at the lowest water calcium treatment level. For a given treatment combination there was a negative correlation between body calcium concentration and body cadmium concentration (egg: r_P = -0.44, p= 0.018; start-feeding larvae: r_P = -0.53, p = 0.001), except for the eyed larvae stage (r_P = -0.01, p= 0.92).

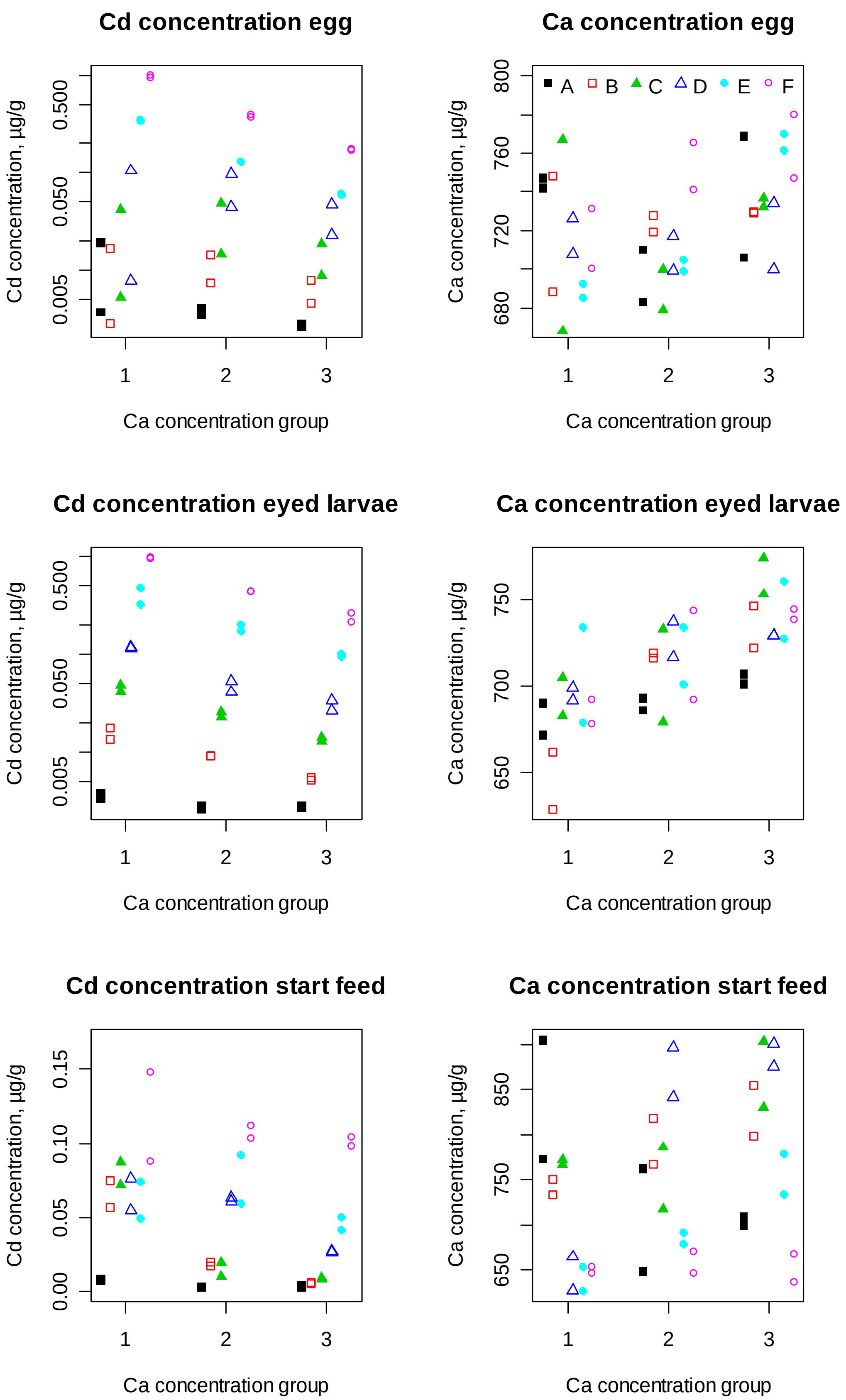


Figure 5. Bivariate plots of concentrations of body Cd and Ca at three life stages. Each point represents concentrations for a mixture of 10 individuals. Concentrations are given as µg Cd per g wet weight of body tissue. Note that the two top left figures have log scaled *y*-axis. Symbol legend in top right figure.

3.5 Biological trait effects

Despite that there was some variation in degreedays among holding tanks this variable was never found to give any significant contribution to any of the trait responses when the ordinary model structure was included (*i.e.*, when estimating type III sum of squares). Also, exclusion or inclusion of Tank 1 (*i.e.*, the coldest tank) did not affect any of the conclusions of the statistical tests. Hence, all results presented in this result section include Tank 1 observations.

3.5.1 Size at stage

The mixed-model GLM analyses revealed that for all size-at-stage traits but egg size (p=0.09) there was a significant interaction effect between cadmium and calcium (table in section 7.1.1 in the Appendix and **Figure 6**). For size at hatch and size at start feeding the full factorial model explained 36% and 48% of the variation, respectively. The explanatory power for the GLM fitted to the two earlier stages were less pronounced (<10%). Egg size and size at eyed stage was larger than control (i.e., group A) at low calcium concentrations, but not so at later life stages. At high calcium concentrations the cadmium effect on size is not that evident as at low concentrations, except that sizes at start feeding the two highest cadmium concentrations produce individuals with the smallest sizes.

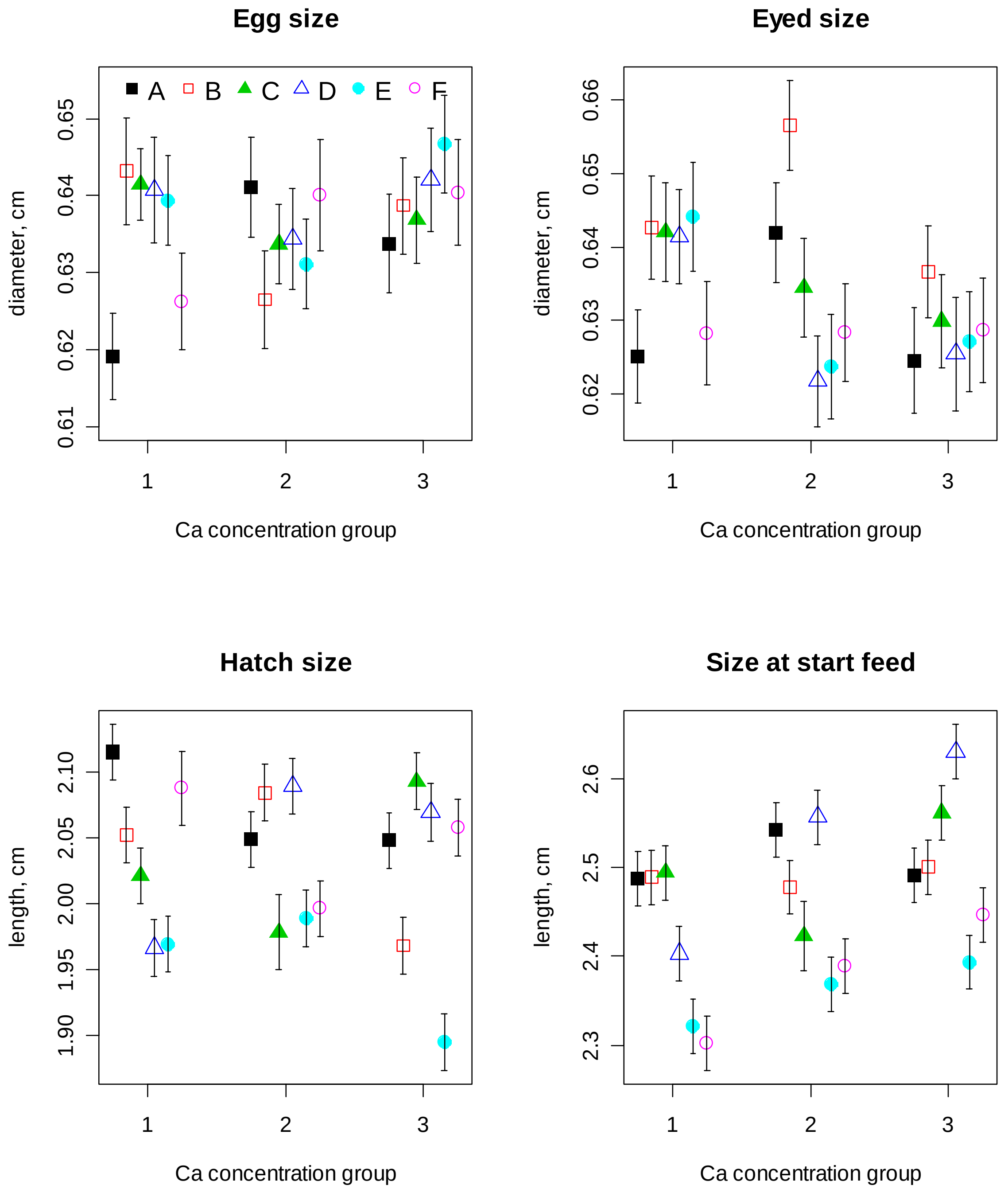


Figure 6. The Least Square Means ± 2 S.E. of four size traits as estimated from mixed model GLMs where replicate variation is accounted for in all models and measurement variation is accounted for in the two egg size traits. Symbol legend in top left figure.

3.5.2 Weight at stage

The mixed-model GLM analyses of the individual weight data had a much lower explanatory power than for the size analyses, ranging from 5% to 23% (table in section 7.1.1 in the Appendix). No strong cadmium vs. calcium interaction effects was revealed, but there was a significant additive effect of cadmium on individual weight for the two later life stages (**Figure 7**). The effect of cadmium was generally negative on the individual weights at low calcium concentrations over all early life stages, except for the eyeing stage. At high calcium concentrations (Ca-level 3), there was no cadmium effect (contrasts: $p>0.05$), except that for the start feeding stage in which the two highest cadmium dosages (E and F) produced individual with significantly smaller weights than the rest of the Cd-groups (contrasts for both levels: $p<0.0001$). The negative effect of high cadmium dosages on individual start feeding weights was pronounced over all calcium levels (all control vs. E and F contrasts: $p< 0.0001$). The maximum difference in weight at start feeding between control group individuals and all the treatment group individuals was 29% (0.111 g vs. 0.157 g).

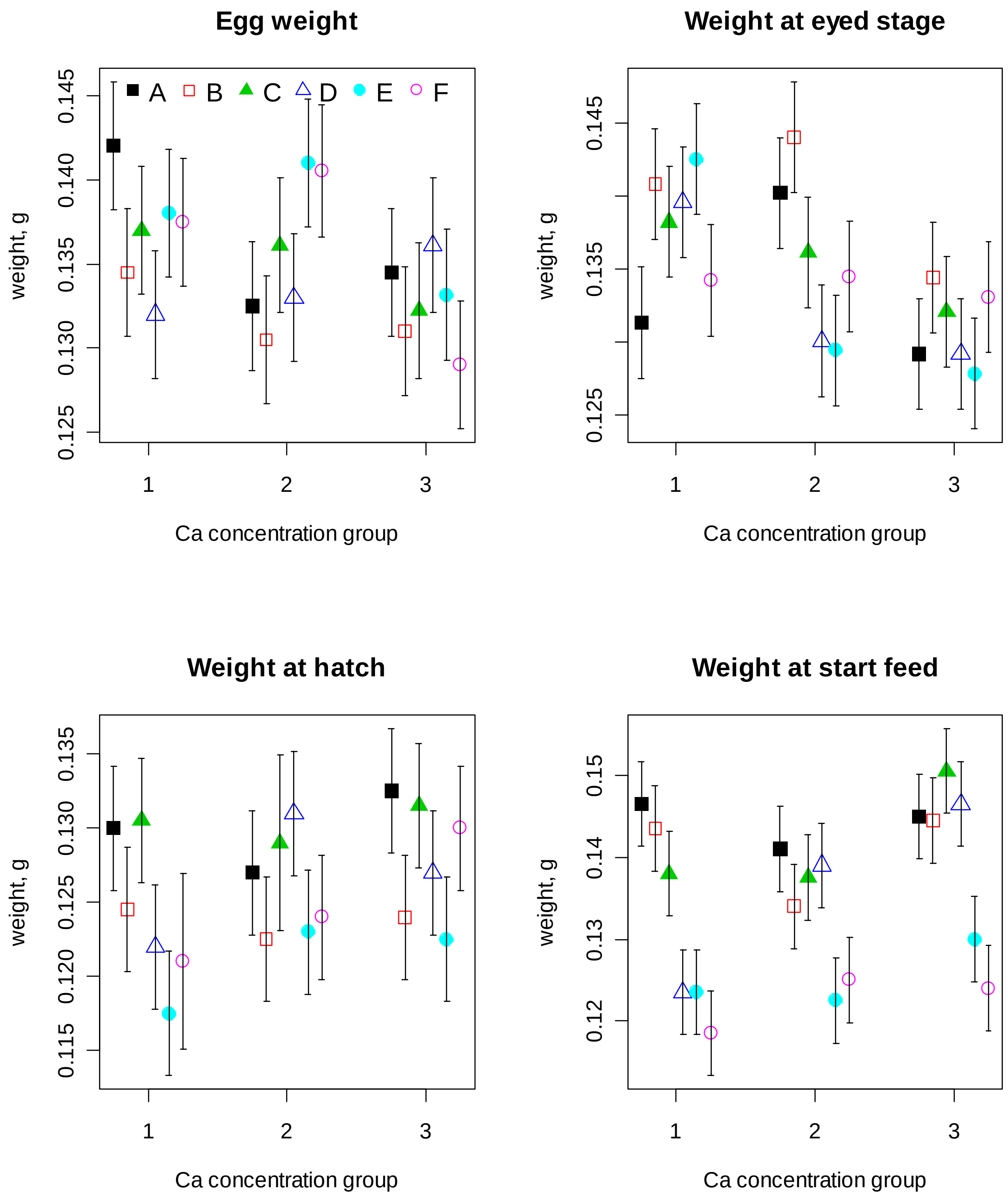


Figure 7. Least square mean plots with corresponding ±2S.E. of individual wet weights as estimated from mixed model GLM using Cd and Ca as fixed predictors and replicates as random effect. There was no indication of degree-day effects for any of the traits modelled. Symbol legend in top left figure

3.5.3 Hatching trajectories

The most supported logistic hatching model revealed that there was no evidence of any Cd *vs.* Ca concentration interaction effect (p>>0.05, see section 7.1.2 in the Appendix for a table). The Akaike Information Criterion (AIC) of Cd vs. Ca concentration interaction effects were always more than 10 units higher than additive models. Furthermore, there was very little support of any additive effect of calcium on the hatching trajectory (AIC more than 12 units higher than models without calcium included as predictor). There was, however, a statistically significant additive effect of cadmium concentration on the hatching trajectory. This effect involved an up to 10 degreedays difference for 50% probability of hatching, where individuals in the control group (i.e., group A) and low Cd concentration groups hatched earlier than high-concentration groups (E and F). Owing to the lack of evidence of degreeday *vs.* cadmium interaction effects on the hatching trajectories the duration of the hatching process was also similar among the cadmium treatment groups.

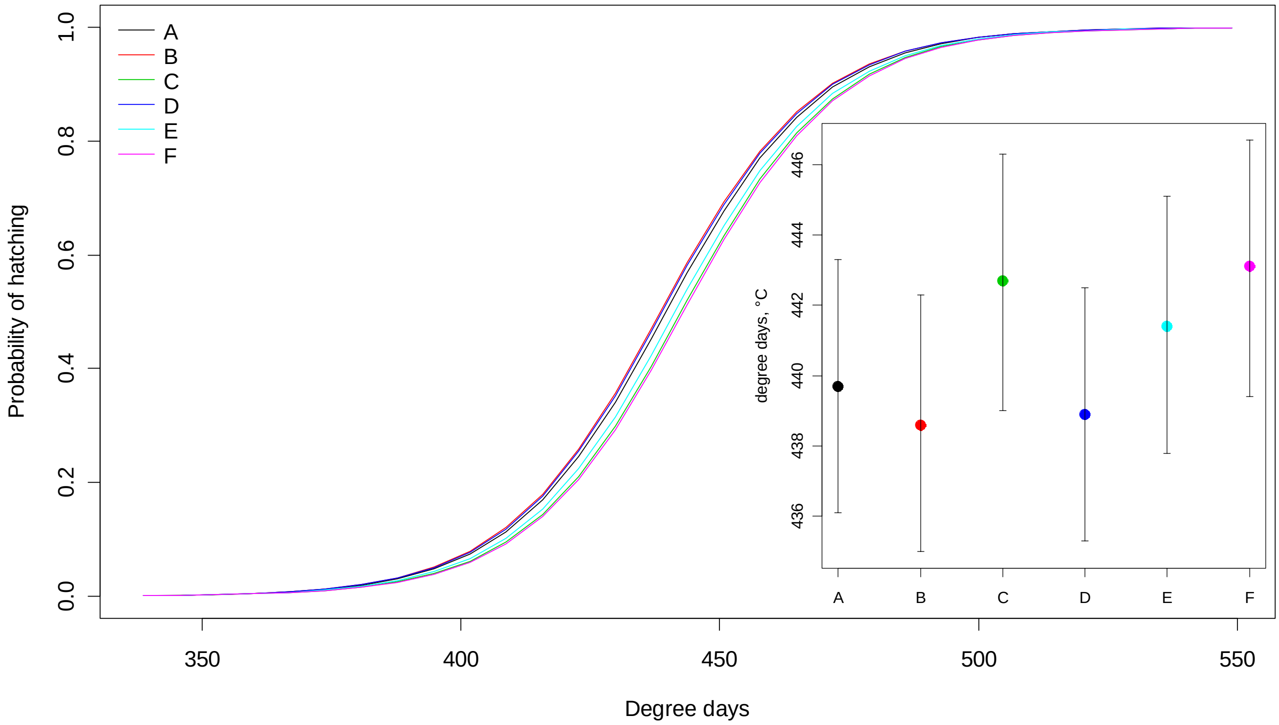


Figure 8. Estimated hatching trajectories as retrieved from binomial GLM-model with additive Cd concentration effect and degree days as predictors. The inserted figure displays estimated 50% probabilities for hatching as function of degree days with corresponding 95% confidence bounds. All probabilities are conditional on that the individuals are going to hatch in the end (*i.e.*, not going to die prior to hatching).

3.5.4 Cumulated mortality

The total mortality was very low over the experimental period (**Table 6**), ranging from 0.5 to 12 %. However, within this range of mortality a Cox model fitted the data revealed that there was a significant calcium *vs.* cadmium concentration interaction effect (p=0.0004, **Table 7**). The estimated cumulated mortality is shown in **Figure 9** and it visualises that the mortality is low throughout the experiment. Hence, estimation of LC_{50} is not possible. Furthermore, the high-dosage levels of cadmium (E and F) have very different response curves, where the E-treatment imposes a higher mortality trajectory than the control in the soft water treatment (contrast: p=0.0003) and not at higher calcium concentrations, whereas the opposite is the case for the F-level dosage (contrast: Ca-level 2: p=0.011, Ca-level 3: p=0.0001).

Table 6. Number of eggs/fry in each group at start and end of exposure. A total of 40 individuals were removed for length and weight determination during the experiment. Mortality during the three defined stages (egg, hatching and fry) was determined individually based on visual and photographical determination of start and end of hatching period. The majority of mortalities during the hatching stage were hatched fry.

Group	Tank No.	Cd-exposure	Hardness	N		Accumulated mortality (%)			
		Cd (µg/L)	CaCO3 (mg/L)	start	End	Egg stage	Hatching	Fry	Total
A1	25-A1	0	0+	206	159	3,0	0,5	0,0	3,5
A1	29-A1	0	0+	224	176	1,9	1,4	0,5	3,7
A2	16-A2	0	10+	224	177	1,4	1,8	0,0	3,2
A2	27-A2	0	10+	231	183	1,8	0,9	0,9	3,6
A3	18-A3	0	40+	247	195	3,0	1,7	0,4	5,1
A3	21-A3	0	40+	259	212	1,6	0,8	0,4	2,8
B1	17-B1	0,1	0+	255	204	1,2	2,9	0,4	4,5
B1	30-B1	0,1	0+	256	202	2,1	3,7	0,0	5,8
B2	10-B2	0,1	10+	244	197	1,3	1,3	0,4	3,0
B2	13-B2	0,1	10+	210	163	2,5	0,0	1,0	3,4
B3	8-B3	0,1	40+	209	166	0,5	1,0	0,0	1,5
B3	34-B3	0,1	40+	255	207	2,0	1,2	0,0	3,2
C1	6-C1	0,3	0+	194	149	2,1	0,5	0,0	2,6
C1	31-C1	0,3	0+	210	160	1,5	2,0	1,5	5,0
C2	1-C2	0,3	10+	178	135	1,7	0,0	0,0	1,7
C2	7-C2	0,3	10+	222	174	0,0	3,7	0,0	3,7
C3	14-C3	0,3	40+	238	192	1,7	0,4	0,4	2,6
C3	32-C3	0,3	40+	197	152	1,0	1,0	0,5	2,6
D1	5-D1	1	0+	174	131	0,6	0,6	0,6	1,8
D1	28-D1	1	0+	240	190	3,9	0,4	0,0	4,3
D2	20-D2	1	10+	225	176	2,8	0,9	0,5	4,2
D2	22-D2	1	10+	255	208	1,2	1,2	0,4	2,8
D3	23-D3	1	40+	262	210	2,8	1,6	0,4	4,8
D3	24-D3	1	40+	215	152	7,3	1,6	3,1	12,0
E1	3-E1	3,2	0+	203	156	3,1	0,5	0,0	3,6
E1	12-E1	3,2	0+	247	192	3,4	2,6	0,4	6,5
E2	26-E2	3,2	10+	214	173	0,5	0,0	0,0	0,5
E2	35-E2	3,2	10+	259	214	1,2	0,0	0,8	2,0
E3	2-E3	3,2	40+	257	213	0,8	0,0	0,8	1,6
E3	4-E3	3,2	40+	203	158	2,0	0,5	0,0	2,5
F1	19-F1	10	0+	216	171	0,9	1,4	0,0	2,4
F1	36-F1	10	0+	241	191	0,9	0,4	3,0	4,3
F2	9-F2	10	10+	221	166	1,5	0,0	5,8	7,3
F2	15-F2	10	10+	243	195	2,6	0,4	0,4	3,4
F3	11-F3	10	40+	220	166	4,4	0,5	1,9	6,8
F3	33-F3	10	40+	235	183	2,2	0,9	2,2	5,4

Table 7. Analysis of deviance table for the Cox survival model: Surv(time,status) = Cd + Ca + Ca*Cd. The model explained 56% of the deviance.

Effect	Df	Deviance	Resid. d.f.	Resid. Deviance	$P(>\|\chi^2\|)$
Cd treatment	5	14.8	7523	5269	0.0113
Ca treatment	2	4.0	7521	5265	0.1000
Cd*Ca treatment	10	32.1	7511	5232.9	0.0004

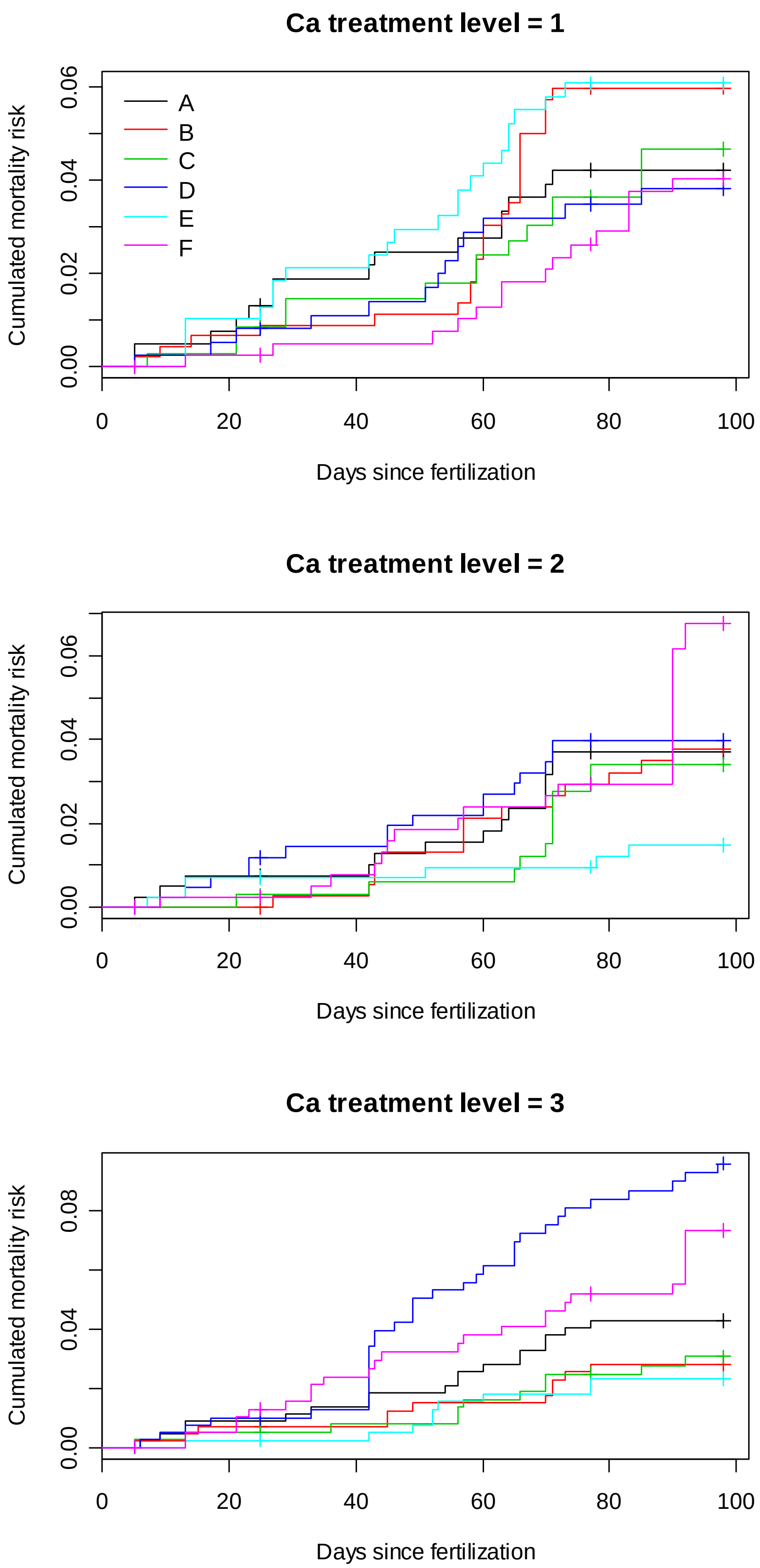


Figure 9. The cumulated mortality risk for the different Cd- and Ca concentration treatments as estimated using the Kaplan-Meier method for a model fitted under a Weibull distribution.

3.5.5 Deformations and abnormal behaviour

In total, very few larvae were deformed. There was no evidence of any heterogeneity in the distribution of these few individuals amongst treatment groups and clearly the low numbers prevented any statistical analysis of these results.

There were few observations of abnormal behaviour other than circular motions observed in relation to some of the deformed larvae. Again, the numbers were too few for statistical analysis.

3.5.6 LOEC, NOEC and EC10 concentrations

Based on the presented biological data and their statistical analyses, we have constructed a table summarising the hardness-specific LOEC and NOEC cadmium values for each trait (**Table 8**). The table shows that the most important findings are the cadmium effects on the size and weight at start feeding. For most other traits the LOECs are either inconclusive due to complex responses (egg diameter), or just the highest cadmium dosage produces significant negative responses (hatching and mortality).

Table 8. $CaCO_3$-specific (given as the averaged values for each treatment level, mg/l) LOEC and NOEC cadmium concentrations (averaged within-treatment concentrations, µg/l) for traits involved in this study. NC indicates non-conclusive analysis (*e.g.*, where there might be effects at lower dosages than NOEC). NA means that there is no LOEC-level, + means all dosages have a positive effect compared to the control. The NOEC slope represents the hardness slope, *i.e.*, slope of ln-transformed toxic threshold to ln transformed measured water hardness level.

	NOEC				LOEC		
Trait / [$CaCO_3$]	2.7	12.8	42.7	slope	2.7	12.8	42.7
Egg diameter	8.5	8.5	8.5	0.00	NC	NC	NC
Eyed egg diameter	8.5	0.31	8.5	-0.10	NA	0.95	NA
Hatch size	8.5	0.95	8.5	-0.07	NC	3.0	NC*
Start feeding size	0.31	0.95	0.14	-0.24	0.95	3.0	3.0**
Egg weight	8.5	8.5	8.5	0.00	NA	NA	NA
Eyed egg weight	8.5	8.5	8.5	0.00	+	NC	NA
Hatch weight	0.95	8.5	8.5	0.82	3.0	NA	NA
Start feeding weight	0.31	0.95	0.95	0.42	0.95	3.0	3.0
Hatching trajectory***		3.0				8.5	
Cumulative mortality	8.5	3.0	3.0	-0.39	NC	8.5	8.5†

* very negative effect for the E-level (p<0.001)
** not significant effect for the F-level (p = 0.11)
*** no effect of calcium, therefore pooled results presented only
† D-level individuals experience higher mortality than control, but E-level response is lower than control.

Weight at start feeding was the only trait demonstrating a fairly consistent negative dose-response relationship. For this trait, we estimated EC10 values from the within-water-hardness linear responses to (averaged over experimental period) cadmium concentrations (**Table 9**). These dose-response relationships were most consistent at low water hardness (R^2 = 0.84), yet significantly negative at all water hardness levels (**Table 9**). The estimated EC10 values with corresponding 95% confidence intervals are displayed in **Figure 10** and from the same figure we see that the EC10 values increase significantly (p<0.05) with increasing $CaCO_3$ concentration, with a hardness slope of 1.03.

Table 9. Linear regression parameters for the dose-response relationship between cadmium and weight at start feeding in trout from Byglandsfjorden. The weight response is scaled so as to setting the average weight values at the control level to 1. Note that the cadmium effect has been estimated on an ln-scale. Do also note that the analyses were performed as weighed regressions where the inverse of the within-treatment trait variance was used as weighing factor.

	Parameter estimate (s.e.)		Model
$CaCO_3$ concentration	Intercept	slope(ln(cadmium))	R^2
2.7 mg/l	0.862 (0.011)***	-0.036 (0.005)***	0.84
12.8 mg/l	0.898 (0.019)***	-0.021 (0.009)*	0.38
42.7 mg/l	0.947 (0.020)***	-0.027 (0.009)*	0.47

* $p<0.05$, *** $p<0.0001$

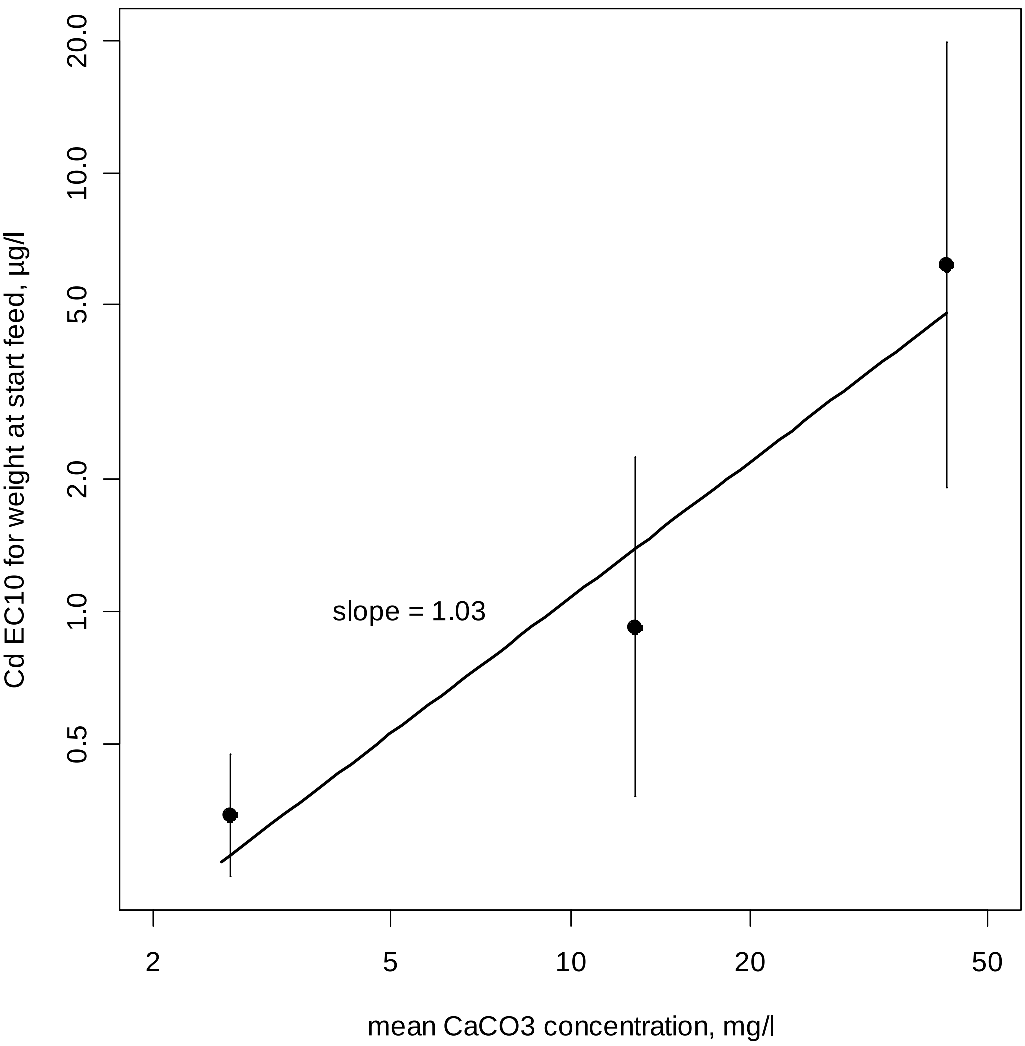


Figure 10. The relationship between estimated cadmium-induced EC10 for weight at start-feeding and water hardness ($CaCO_3$ concentration). Error bars represent 2SE for the estimated EC10. The slope represents the estimated slope for the effect of $CaCO_3$ on EC10. Note that both axes are log-scaled.

4. Discussion

4.1 Experimental protocol

The experimental protocol gave reasonably stable conditions during the experimental period in terms of dilution water chemistry (**Table 3**), exposure concentrations of Ca and Cd (**Table 4**), and measured oxygen saturation above 90% saturation at all times. Temperature showed some variation due to climatic conditions (**Table 5**), and some between-tank variation due to placement in the experimental facility (**Table 5**, **Figure 4**). However, this inter-tank variation in temperature did not confound the main effects tested in the experiment. Incubation temperature was within the recommended interval for salmonids (Bæverfjord et al. 1998). The fertilization and incubation procedure was very successful based on determination at the 2-4 cell stage, as well as low mortality rates in most experimental groups during the entire experiment, and very few observed malformed larvae. Hence, the experiment was conducted according to the OECD Guideline 210, Fish Early Life Stage Test with no vital deviations.

4.2 Body cadmium and calcium concentrations

Cadmium uptake in eggs and larvae of fish is reported to be influenced by pH, with increased accumulation at all Cd concentrations at pH below 6.0 (Peterson et al. 1985). The level of humic acids in the water only slightly influence Cd uptake (Hammock et al. 2003). The water quality in the reported experiment had pH well above 6.0, and low humic levels (**Table 3**) that very unlikely influenced Cd toxicity.

When Cd is accumulated in fish eggs, it may be detected in the different components at different concentrations. Typically most of the Cd is associated with the chorion (Peterson and Martin-Robichaud, 1986). In salmonids like rainbow trout (*Onchorhynchus mykiss*) and chinook salmon (*O. tshawytscha*), more than 93% of the total Cd absorbed by the egg is retained in the chorion (Beattie and Pascoe, 1978, Hammock et al., 2003). This study did not separate metal concentration measurements from different parts of the egg, but the observed tremendous drop in total body Cd concentrations between eyed egg stage and start feeding larvae (for the F-treatment the drop was, on average, from 0.97 to 0.16 mg Cd/g body tissue, **Figure 5**) clearly indicates that at least 85% of pre hatch total body Cd is accumulated in the chorion.

In accordance with other researchers (*e.g.*, Wu et al. 2007), we find that start-feeding larvae seem more able to modulate the body Cd concentration at higher water hardness levels (**Figure 5**). Cd^{2+} has been found to compete with Ca^{2+} for the Ca^{2+}-channel in cells, but a previous study found that tilapia larvae (*Oreochromis mossambicus*) could rapidly modulate their Ca^{2+} uptake in the presence of Cd^{2+} (Chang et al. 1997). Clearly, the brown trout start feeding larvae have limitations to their modulation efficiency at water Cd concentrations above 3 µg/l (E-treatment) as they are not able to down-scale the body Cd concentration even at water hardness levels of 42 mg $CaCO_3$/l. At water hardness values of 2.7 mg $CaCO_3$/l the brown trout start feeders are not able to modulate body Cd concentrations at water concentrations of 0.14 µg Cd/l. Hence, this experiment demonstrates that body cadmium concentration in very early post-hatch life stages indeed is linked to water hardness.

4.3 Biological effects

4.3.1 Size and weight effects

This study has documented that the brown trout traits size (*i.e.*, length) and weight at start feeding are the most sensitive early-life traits towards cadmium concentrations above 0.95 to 3.0 µg/l (**Table 8**). Consequently, owing to the similar initial egg sizes among treatment groups, we have documented that egg-to-start-feeding growth has been significantly impeded by effects of cadmium. Furthermore, the effect of cadmium on growth was most pronounced at the lowest water hardness levels (**Figure 6** and **Figure 7**), resulting in a significant cadmium×calcium interaction effect.

The high sensitivity of early-life growth towards cadmium has been documented for several other fish species as well. Inhibition of growth was determined by Rombough and Garside (1982) as the most sensitive indicator of Cd exposure in Atlantic salmon larvae, with significant effects at 0.47 µg Cd/l at water Ca levels 5.5 mg Ca/l. Similar results have recently been published by Miliou (1998) and Wu et al (2007) for guppy and tilapia larvae, respectively. Rose et al. (2006) also documented that the smallest larval topsmelt (*Atherinops affinis*) individuals were found in the highest Cd treatment (50 and 100 ppb Cd), and interestingly, they documented that these fish were respiring at higher rates than control fish. They found that higher oxygen consumption rates of Cd-exposed topsmelt were associated with reduced growth, indicating that less energy was allocated for growth because of an increased metabolic demand for detoxification or elimination of Cd.

In accordance with our findings, Wu et al. (2007) document that high water hardness contributes to decreased growth inhibition of cadmium. They suggest that Ca^{2+} helps maintain internal ionic homeostasis and thus contribute to decreased growth inhibition by Cd. It has been well documented that Ca^{2+} competes Ca^{2+} channel with Cd^{2+}, and a high ambient Ca^{2+} concentration would decrease Cd^{2+} toxicity to aquatic animals (Guven et al. 1995).

4.3.2 Hatching trajectories

This study documented a significant additive effect of water cadmium concentration on hatching trajectory (**Figure 8**) with little support to eventual effects of water hardness. Even though there was a statistically significant effect, the effect was negligible in biological terms. A maximum difference in 50% hatching probability of 10 degreedays is rather minor as we are then talking about 1.5–2 days. This conclusion is also in accordance with findings in Rombough and Garside (1982). They documented no significant effect on developmental rate or interval to 50 % hatch in the concentration range 2.8-870 µg Cd/l for Atlantic salmon (*S. salar*).

4.3.3 Cumulated mortality

As for the hatching trajectory, we documented significant effects of cadmium water concentration on cumulative mortality trajectories (**Figure 9**). The mortality level, however, was very low, not exceeding 12% in any replicate unit. Furthermore, the highly significant cadmium×calcium interaction effect produced non-consistent mortality responses to the cadmium treatment across water hardness levels. The mortality results in this experiment is therefore not conclusive, from a biological perspective, but it can be concluded that early-life mortality in brown trout is insensitive towards water Cd concentrations below 9 µg/l at water hardness below 42 mg $CaCO_3$/l. For Atlantic salmon, estimates on LC_{50} from fertilization to hatch were assessable at cadmium concentrations beyond 300 µg Cd/l, but increased sensitivity was found at later alevine stages, with significant mortalities at 8,2 µg Cd/l (Rombough and Garside, 1982).

There are good reasons for suspecting acute mortality effects of cadmium in fish as this element has many pathways of reactions that can cause detrimental physiological responses. Cadmium can damage gills and decreases the activity of gill Ca^{2+}-ATPase, which leads to fish hypocalcemia (Wong & Wong 2000) and can result in skeletal deformities and disturbed Ca balance (Wicklund-Glynn et al. 1994). In addition, Cd has adverse effects on growth, reproduction, respiratory functions, and osmoregulation (Pratap & Wendelaar Bonga 1990).

Based on the findings in this study, and also in Rombough and Garside (1982), it seems likely that mortality costs from exposure to cadmium are more likely to occur at later life stages than covered by this study.

4.4 Implications for the risk assessment of cadmium

We found that NOEC for the most sensitive trait, weight at start feeding, is 0.31 µg Cd/l. With the estimated hardness slope of 0.42 µg Cd/l per mg $CaCO_3$/l the prevailing regional PNEC at 0.08 µg Cd/l seems sufficiently protective for early life stages of trout even at water hardness values lower than 2.7 $CaCO_3$.

As a final remark, we want to emphasize that reports on pronounced survival and growth effects of cadmium at post start-feeding life stages pinpoints the necessity to conduct soft-water studies on these life stages in the future.

5. Conclusions

- The experiment was conducted according to the OECD Guideline 210, Fish Early Life Stage Test with no vital deviations.
- Body concentrations of Cd correspond to water concentrations up at hatching, after which there is a relatively higher level of Cd in body tissue at low water hardness compared to high water hardness
- The was no evidence of vital biological effects of Cd on pre-hatching life stages
- The was no consistent evidence of increased egg-to-start-feed mortality due to Cd at any water hardness level
- The was no vague evidence of delayed hatching at very high Cd concentrations (*i.e.*, LOEC = 8.5 µg Cd/l)
- The was consistent evidence that size and weight at start feed is impeded at Cd concentrations equal to and higher than 3.0 µg Cd/l for all hardness levels with increasing Cd sensitivity at decreasing hardness levels
- Because reduced size at start feeding coincide with high body concentrations of Cd there is reasonable to link the growth reduction to negative influence of Cd on somatic growth
- NOEC for the most sensitive trait, weight at start feeding, is estimated at 0.31 µg Cd/l. With the estimated hardness slope of 0.42 µg Cd/l per mg $CaCO_3$/l at hand we can conclude that the prevailing regional PNEC at 0.08 µg Cd/l seems sufficiently protective for early life stages of trout even at water hardness values lower than 2.7 mg $CaCO_3$/l.
- Owing to reports on pronounced survival and growth effects of cadmium at post startfeeding life stages soft-water studies on these life stages should be performed in the future

6. References

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7. Appendices

7.1 Statistical tables

7.1.1 Size and weight at stage analyses

	Model					Effect test					
	df						df				
Trait	Num	Den	F	p	R^2	Source	Num	Den	SS	F	p
Egg size	18	354	1.399	0.12	0.07	Cd treat	5	336	0.213	0.66	0.649
						Ca-treat	2	336	0.167	1.30	0.272
						Cd*Ca-treat	10	336	1.066	1.66	0.088
						Replicate_R	1	336	0.044		
Size at eyed egg	18	359	2.562	***	0.13	Cd treat	5	341	1.072	3.22	**
						Ca-treat	2	341	0.374	2.81	0.061
						Cd*Ca-treat	10	341	1.270	1.91	*
						Replicate_R	1	341	0.206		
Size at hatch	18	337	9.660	***	0.36	Cd treat	5	319	0.495	14.95	***
						Ca-treat	2	319	0.010	0.78	0.455
						Cd*Ca-treat	10	319	0.623	9.40	***
						Replicate_R	1	319	0.038		
Size at start-feeding	18	358	17.32	***	0.48	Cd treat	5	340	1.376	26.70	***
						Ca-treat	2	340	0.974	47.28	***
						Cd*Ca-treat	10	340	0.766	7.43	***
						Replicate_R	1	340	0.104		
Egg weight	18	351	0.91	0.56	0.05	Cd treat	5	333	0.001	0.76	0.575
						Ca-treat	2	333	0.001	1.84	0.158
						Cd*Ca-treat	10	333	0.003	0.87	0.561
						Replicate_R	1	333	0.000		
Weight at eyed egg	18	359	1.83	*	0.09	Cd treat	5	341	0.002	1.46	0.202
						Ca-treat	2	341	0.003	5.40	**
						Cd*Ca-treat	10	341	0.004	1.36	0.193
						Replicate_R	1	341	0.000		
Weight at hatch	18	339	0.95	0.51	0.05	Cd treat	5	321	36.23	2.06	*
						Ca-treat	2	321	7.460	1.06	0.346
						Cd*Ca-treat	10	321	15.46	0.44	0.925
						Replicate_R	1	321	0.000		
Weight at start-feeding	18	359	5.21	***	0.23	Cd treat	5	341	0.025	12.37	***
						Ca-treat	2	341	0.004	5.44	**
						Cd*Ca-treat	10	341	0.007	1.50	0.134
						Replicate_R	1	341	0.003		

* $p<0.05$, ** $p<0.001$, *** $p< 0.0001$

7.1.2 Hatching trajectory analyses

				Residual			Fit statistics	
Model #	Source	Df	Deviance	df	Deviance	$p>\|\chi^2\|$	Deviance explained	AIC
1	Cd-treat	5	20.13	1074	981.34	***	0.833	416.19
	Ca-treat	1	1.02	1073	980.32	0.31		
	Degree-days	1	800.97	1072	179.35	****		
	Cd-treat:Ca-treat	5	3.51	1067	175.84	0.62		
	Cd-treat:degree-days	5	2.63	1062	173.21	0.76		
	Ca-treat:degree-days	1	0.58	1061	172.63	0.45		
	Cd-treat:Ca-treat:degree-days	5	5.41	1056	167.22	0.37		
2	Cd-treat	5	20.13	1074	981.34	***	0.824	394.92
	Ca-treat	1	1.02	1073	980.32	0.31		
	degree-days	1	800.97	1072	179.35	****		
	Cd-treat:Ca-treat	5	3.51	1067	175.84	0.62		
3	Cd-treat	5	20.13	1074	981.34	***	0.819	**387.3**
	degree-days	1	800.21	1073	181.13	****		

*** $p < 0.0001$, **** $p < 1*10^{-10}$

7.2 Raw data

7.2.1 Egg diameter

Provided as mean individual egg diameters in cm (left number) and corresponding standard deviations (right numbers) for four replicate measurements per individual.

Tank	Treat-	Individual number																			
No	ment	No 1		No 2		No 3		No 4		No 5		No 6		No 7		No 8		No 9		No 10	
1	C2	0.64	0.03	0.64	0.02	0.63	0.00	0.64	0.02	0.62	0.01	0.64	0.00	0.62	0.01	0.61	0.01	0.64	0.01	0.59	0.01
2	E3	0.62	0.02	0.63	0.01	0.67	0.02	0.70	0.01	0.68	0.01	0.67	0.01	0.66	0.02	0.68	0.02	0.68	0.01	0.68	0.02
3	E1	0.59	0.01	0.60	0.02	0.64	0.01	0.65	0.02	0.63	0.02	0.69	0.02	0.62	0.01	0.63	0.01	0.63	0.01	0.63	0.02
4	E3	0.64	0.02	0.61	0.02	0.69	0.01	0.62	0.03	0.62	0.00	0.61	0.02	0.62	0.00	0.63	0.02	0.68	0.01	0.63	0.02
5	D1	0.63	0.02	0.62	0.02	0.63	0.01	0.60	0.02	0.64	0.02	0.61	0.03	0.65	0.02	0.62	0.04	0.64	0.01	0.65	0.02
6	C1	0.60	0.01	0.63	0.02	0.60	0.01	0.65	0.03	0.66	0.00	0.64	0.01	0.64	0.01	0.65	0.01	0.65	0.00	0.65	0.01
7	C2	0.69	0.02	0.66	0.02	0.61	0.03	0.67	0.02	0.61	0.02	0.66	0.01	0.62	0.02	0.60	0.02	0.63	0.02	0.65	0.03
8	B3	0.66	0.04	0.60	0.01	0.64	0.01	0.62	0.01	0.63	0.01	0.63	0.01	0.69	0.02	0.63	0.02	0.58	0.01	0.67	0.01
9	F2	0.63	0.02	0.64	0.01	0.62	0.01	0.65	0.02	0.60	0.02	0.66	0.03	0.63	0.03	0.63	0.01	0.69	0.01	0.62	0.02
10	B2	0.63	0.01	0.61	0.02	0.62	0.02	0.61	0.01	0.68	0.01	0.60	0.02	0.62	0.01	0.61	0.01	0.59	0.02	0.63	0.02
11	F3	0.64	0.03	0.64	0.02	0.66	0.03	0.61	0.01	0.62	0.01	0.63	0.03	0.69	0.02	0.63	0.01	0.63	0.01	0.66	0.02
12	E1	0.66	0.01	0.63	0.01	0.65	0.02	0.62	0.01	0.64	0.03	0.69	0.00	0.64	0.00	0.63	0.01	0.61	0.01	0.59	0.01
13	B2	0.61	0.01	0.57	0.02	0.60	0.01	0.61	0.01	0.57	0.01	0.67	0.01	0.66	0.02	0.65	0.01	0.62	0.03	0.67	0.01
14	C3	0.69	0.02	0.67	0.01	0.68	0.02	0.62	0.01	0.62	0.01	0.68	0.02	0.65	0.02	0.63	0.01	0.61	0.01	0.60	0.03
15	F2	0.65	0.02	0.60	0.01	0.63	0.02	0.66	0.04	0.59	0.04	0.62	0.02	0.63	0.01	0.67	0.01	0.68	0.03	0.69	0.03
16	A1	0.68	0.01	0.62	0.02	0.58	0.01	0.63	0.02	0.64	0.01	0.68	0.01	0.61	0.01	0.66	0.02	0.64	0.01	0.65	0.01
17	B1	0.63	0.01	0.63	0.01	0.62	0.01	0.62	0.01	0.63	0.01	0.65	0.01	0.68	0.02	0.66	0.02	na	na	na	na
18	A3	0.68	0.01	0.66	0.02	0.61	0.02	0.63	0.03	0.63	0.01	0.63	0.01	0.65	0.02	0.63	0.02	0.61	0.01	0.63	0.03
19	F1	0.61	0.01	0.59	0.01	0.66	0.01	0.62	0.01	0.58	0.02	0.69	0.02	0.63	0.00	0.63	0.02	na	na	na	na
20	D2	0.61	0.01	0.66	0.01	0.62	0.01	0.60	0.02	0.64	0.01	0.61	0.01	0.65	0.02	0.62	0.02	0.64	0.01	0.64	0.01
21	A3	0.64	0.01	0.64	0.02	0.59	0.01	0.63	0.01	0.62	0.03	0.60	0.01	0.68	0.03	0.64	0.02	0.64	0.01	0.66	0.00
22	D2	0.65	0.01	0.64	0.03	0.60	0.01	0.62	0.02	0.62	0.03	0.61	0.01	0.68	0.03	0.63	0.01	0.64	0.01	0.66	0.01
23	D3	0.62	0.01	0.63	0.02	0.65	0.02	0.64	0.01	0.62	0.01	0.65	0.02	0.66	0.01	0.65	0.02	0.65	0.03	0.68	0.01
24	D3	0.63	0.02	0.65	0.01	0.68	0.01	0.62	0.02	0.63	0.01	0.67	0.02	0.67	0.02	0.62	0.02	0.63	0.01	0.61	0.02
25	A1	0.58	0.00	0.64	0.02	0.67	0.02	0.64	0.04	0.65	0.02	0.58	0.01	0.59	0.00	0.65	0.01	0.67	0.01	0.63	0.01
26	E2	0.65	0.03	0.67	0.01	0.68	0.01	0.68	0.01	0.60	0.01	0.64	0.03	0.68	0.02	0.58	0.01	0.61	0.00	0.66	0.02
27	A2	0.64	0.02	0.62	0.01	0.65	0.01	0.67	0.02	0.66	0.02	0.65	0.03	0.64	0.02	0.66	0.02	0.61	0.02	0.64	0.01
28	D1	0.64	0.02	0.69	0.02	0.62	0.01	0.62	0.01	0.62	0.01	0.68	0.01	0.68	0.02	0.68	0.02	0.65	0.01	0.67	0.01
29	A1	0.66	0.01	0.66	0.01	0.59	0.01	0.67	0.02	0.61	0.02	0.65	0.02	0.67	0.01	0.60	0.01	0.61	0.03	0.63	0.01
30	B1	0.62	0.02	0.67	0.02	0.67	0.01	0.65	0.01	0.64	0.02	0.63	0.02	0.64	0.04	0.62	0.01	0.66	0.02	0.68	0.01
31	C1	0.64	0.01	0.59	0.00	0.61	0.02	0.66	0.03	0.68	0.01	0.65	0.01	0.64	0.02	0.65	0.00	0.59	0.01	0.69	0.01
32	C3	0.66	0.00	0.62	0.00	0.64	0.01	0.67	0.01	0.61	0.01	0.64	0.02	0.62	0.00	0.64	0.02	0.63	0.02	0.63	0.03
33	F3	0.63	0.02	0.65	0.01	0.63	0.02	0.62	0.03	0.68	0.03	0.61	0.01	0.64	0.02	0.68	0.02	0.67	0.01	na	na
34	B3	0.66	0.02	0.63	0.01	0.65	0.01	0.66	0.03	0.66	0.03	0.62	0.02	0.63	0.02	0.68	0.02	0.64	0.01	0.67	0.01
35	E2	0.61	0.02	0.62	0.01	0.65	0.01	0.62	0.02	0.61	0.01	0.64	0.02	0.61	0.01	0.63	0.03	0.66	0.01	0.67	0.01
36	F1	0.60	0.01	0.67	0.01	0.61	0.01	0.65	0.02	0.62	0.01	0.56	0.01	0.60	0.02	0.62	0.02	0.65	0.01	0.63	0.02

7.2.2 Diameter at eyeing stage

Provided as mean individual egg diameters in cm (left number) and corresponding standard deviations (right numbers) for four replicate measurements per individual.

Tank		Individual No																			
No	Treat-ment	No 1		No 2		No 3		No 4		No 5		No 6		No 7		No 8		No 9		No 10	
1	C2	0.67	0.01	0.62	0.01	0.59	0.01	0.58	0.01	0.65	0.01	0.63	0.01	0.65	0.02	0.63	0.02	0.66	0.01	0.64	0.01
2	E3	0.61	0.03	0.61	0.01	0.59	0.02	0.62	0.02	0.62	0.01	0.61	0.02	0.66	0.00	0.63	0.01	0.66	0.01	0.63	0.01
3	E1	0.68	0.02	0.66	0.03	0.68	0.02	0.64	0.01	0.65	0.01	0.65	0.01	0.70	0.02	0.65	0.01	0.68	0.01	0.67	0.03
4	E3	0.59	0.04	0.63	0.01	0.63	0.02	0.60	0.02	0.61	0.03	0.57	0.01	0.62	0.02	0.61	0.01	0.66	0.01	0.63	0.01
5	D1	0.62	0.01	0.66	0.02	0.65	0.01	0.63	0.01	0.70	0.01	0.63	0.02	0.69	0.02	0.58	0.03	0.67	0.02	0.63	0.02
6	C1	0.60	0.01	0.63	0.02	0.67	0.02	0.68	0.01	0.65	0.01	0.62	0.02	0.62	0.01	0.63	0.02	0.64	0.02	0.68	0.00
7	C2	0.66	0.01	0.66	0.01	0.62	0.01	0.61	0.01	0.66	0.02	0.61	0.01	0.62	0.01	0.68	0.01	0.63	0.01	0.61	0.02
8	B3	0.61	0.01	0.60	0.01	0.63	0.04	0.61	0.01	0.68	0.01	0.67	0.01	0.64	0.02	0.62	0.02	0.68	0.02	0.58	0.01
9	F2	0.67	0.02	0.60	0.01	0.67	0.02	0.63	0.02	0.59	0.01	0.67	0.03	0.62	0.02	0.63	0.01	0.63	0.02	0.62	0.01
10	B2	0.60	0.02	0.68	0.01	0.65	0.03	0.68	0.00	0.67	0.01	0.63	0.03	0.60	0.02	0.59	0.01	0.68	0.00	0.62	0.01
11	F3	0.58	0.01	0.67	0.01	0.60	0.02	0.62	0.01	0.62	0.03	0.66	0.01	0.62	0.01	0.63	0.02	0.60	0.01	0.65	0.02
12	E1	0.64	0.02	0.60	0.03	0.65	0.03	0.64	0.03	0.65	0.01	0.59	0.01	0.65	0.02	0.59	0.02	0.66	0.01	0.58	0.01
13	B2	0.65	0.03	0.66	0.02	0.65	0.01	0.62	0.03	0.64	0.00	0.63	0.01	0.61	0.02	0.68	0.01	0.69	0.01	0.65	0.04
14	C3	0.63	0.02	0.61	0.02	0.61	0.01	0.63	0.02	0.64	0.01	0.68	0.03	0.68	0.01	0.65	0.01	0.68	0.01	0.62	0.01
15	F2	0.64	0.01	0.63	0.01	0.61	0.01	0.59	0.02	0.65	0.00	0.61	0.01	0.70	0.02	0.61	0.01	0.61	0.02	0.65	0.01
16	A1	0.62	0.01	0.65	0.01	0.62	0.02	0.64	0.01	0.63	0.01	0.64	0.00	0.65	0.01	0.65	0.02	0.69	0.01	0.64	0.03
17	B1	0.63	0.04	0.59	0.03	0.61	0.01	0.65	0.04	0.65	0.02	0.64	0.01	0.64	0.01	0.64	0.01	0.69	0.01	0.64	0.01
18	A3	0.57	0.02	0.60	0.01	0.60	0.02	0.58	0.02	0.64	0.01	0.63	0.02	0.59	0.01	0.66	0.01	0.65	0.02	0.63	0.01
19	F1	0.60	0.01	0.61	0.02	0.59	0.02	0.66	0.01	0.62	0.00	0.69	0.03	0.66	0.01	0.57	0.02	0.61	0.02	0.67	0.01
20	D2	0.63	0.01	0.63	0.02	0.67	0.01	0.62	0.01	0.64	0.03	0.62	0.01	0.64	0.01	0.64	0.00	0.65	0.01	0.59	0.01
21	A3	0.59	0.01	0.68	0.01	0.61	0.03	0.62	0.01	0.61	0.02	0.67	0.02	0.65	0.01	0.62	0.01	0.68	0.03	0.61	0.02
22	D2	0.67	0.01	0.61	0.01	0.61	0.01	0.62	0.01	0.60	0.01	0.62	0.01	0.61	0.01	0.62	0.01	0.61	0.04	0.58	0.00
23	D3	0.58	0.01	0.63	0.02	0.61	0.02	0.64	0.02	0.65	0.01	0.65	0.01	0.64	0.01	0.61	0.02	0.63	0.02	0.62	0.02
24	D3	0.66	0.01	0.65	0.03	0.60	0.02	0.59	0.03	0.59	0.02	0.58	0.02	0.62	0.02	0.60	0.02	0.67	0.01	0.60	0.02
25	A1	0.62	0.01	0.61	0.01	0.65	0.02	0.63	0.02	0.65	0.01	0.62	0.01	0.61	0.03	0.65	0.01	0.63	0.01	0.60	0.01
26	E2	0.57	0.01	0.61	0.03	0.61	0.01	0.64	0.01	0.61	0.02	0.67	0.02	0.61	0.02	0.62	0.03	0.66	0.02	0.58	0.02
27	A2	0.66	0.02	0.67	0.02	0.58	0.01	0.67	0.01	0.64	0.02	0.65	0.02	0.69	0.02	0.63	0.02	0.62	0.01	0.62	0.02
28	D1	0.65	0.02	0.65	0.00	0.64	0.02	0.62	0.01	0.58	0.01	0.65	0.03	0.64	0.00	0.59	0.02	0.66	0.02	0.63	0.01
29	A1	0.61	0.01	0.63	0.01	0.66	0.01	0.62	0.02	0.63	0.01	0.63	0.00	0.58	0.02	0.65	0.02	0.61	0.01	0.60	0.02
30	B1	0.69	0.02	0.61	0.02	0.64	0.02	0.62	0.01	0.67	0.00	0.62	0.02	0.69	0.02	0.63	0.02	0.62	0.01	0.62	0.01
31	C1	0.62	0.02	0.64	0.02	0.65	0.01	0.67	0.01	0.62	0.01	0.60	0.01	0.67	0.01	0.58	0.01	0.63	0.01	0.68	0.02
32	C3	0.61	0.02	0.62	0.01	0.61	0.03	0.62	0.01	0.63	0.01	0.61	0.00	0.61	0.01	0.60	0.01	0.63	0.02	0.65	0.01
33	F3	0.63	0.03	0.60	0.01	0.63	0.01	0.60	0.02	0.62	0.03	0.60	0.01	0.64	0.02	0.65	0.02	0.64	0.03	0.69	0.01
34	B3	0.65	0.01	0.70	0.00	0.62	0.01	0.63	0.03	0.60	0.03	0.64	0.03	0.66	0.01	0.64	0.00	0.60	0.00	0.59	0.02
35	E2	0.62	0.01	0.65	0.02	0.61	0.01	0.62	0.01	0.65	0.01	0.63	0.01	0.63	0.03	0.64	0.01	0.64	0.01	0.62	0.01
36	F1	0.65	0.02	0.63	0.03	0.63	0.02	0.60	0.01	0.61	0.02	0.63	0.02	0.65	0.04	0.63	0.01	0.64	0.01	0.61	0.02

7.2.3 Length at hatch

Provided as individual lengths (cm).

Tank No	Treatment	Individual number									
1	C2	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
2	E3	1.929	1.764	1.838	1.912	1.808	1.998	1.867	1.882	1.856	1.831
3	E1	1.841	1.945	1.930	1.917	1.935	1.880	2.003	1.850	1.875	1.895
4	E3	1.900	1.966	1.914	2.010	1.902	1.972	1.850	1.965	1.827	1.914
5	D1	1.914	1.897	1.936	1.984	1.897	1.943	1.925	1.849	1.905	1.976
6	C1	1.916	1.993	1.922	1.971	1.992	1.843	1.970	1.917	1.964	1.897
7	C2	2.016	2.103	1.990	1.915	1.922	2.040	1.977	2.014	1.935	1.975
8	B3	2.038	1.998	1.913	2.034	1.937	1.975	1.860	1.946	1.944	2.037
9	F2	1.962	2.083	1.966	1.950	1.957	1.979	1.933	1.886	2.023	2.076
10	B2	2.000	1.986	2.035	2.009	2.046	2.045	2.013	2.024	2.030	1.793
11	F3	1.961	1.930	2.134	2.051	2.182	2.104	1.932	2.032	1.917	2.110
12	E1	2.040	2.103	2.156	2.024	1.969	2.089	2.008	2.005	2.014	1.909
13	B2	2.127	2.206	2.174	2.080	2.077	2.166	2.077	2.283	2.056	2.181
14	C3	2.034	2.195	2.021	2.219	2.067	2.073	2.067	2.196	2.102	2.102
15	F2	1.999	2.137	1.929	2.007	1.883	2.052	2.072	1.985	2.070	1.983
16	A1	2.097	2.031	2.018	2.150	2.201	2.046	2.053	1.927	2.022	2.065
17	B1	2.158	2.146	2.110	2.166	1.953	2.102	2.035	2.063	2.125	1.952
18	A3	2.075	2.089	2.172	2.025	2.029	1.979	2.002	2.083	2.013	2.058
19	F1	2.214	2.095	2.025	1.969	2.131	1.988	2.265	2.047	2.014	2.028
20	D2	2.049	2.074	2.208	1.950	2.085	2.039	2.062	2.080	2.097	2.139
21	A3	2.063	2.026	2.049	1.989	1.964	1.916	2.051	2.089	2.193	2.099
22	D2	2.233	2.017	2.137	2.150	2.048	2.090	2.083	2.103	2.081	2.067
23	D3	2.026	2.086	1.982	2.116	2.201	2.083	1.973	2.093	1.988	na
24	D3	2.185	2.130	2.081	2.001	2.140	2.123	2.054	1.927	2.161	1.982
25	A1	2.077	2.280	2.016	2.290	2.097	2.089	2.020	2.090	2.039	2.195
26	E2	1.957	2.062	2.160	2.119	2.028	1.923	2.231	2.101	2.036	2.062
27	A2	2.073	2.029	2.143	2.058	1.861	1.928	2.046	2.158	2.049	2.026
28	D1	2.027	1.946	2.025	2.000	1.891	1.972	1.838	2.084	2.109	2.218
29	A1	2.144	2.156	2.167	2.028	2.155	1.964	2.155	2.159	2.133	2.051
30	B1	1.935	1.910	2.122	1.908	2.047	2.095	2.067	2.110	1.995	2.054
31	C1	2.015	2.101	2.033	2.061	2.266	2.175	2.087	2.066	2.050	2.185
32	C3	2.026	2.076	2.211	2.065	1.992	2.149	2.040	2.034	2.141	2.056
33	F3	2.088	2.142	2.134	2.063	2.090	2.069	2.088	2.029	2.091	2.014
34	B3	2.011	2.051	2.073	1.807	2.002	2.031	1.968	1.930	1.744	2.068
35	E2	1.856	1.953	1.859	1.941	1.932	1.969	1.958	1.937	1.847	1.848
36	F1	na	na	na	na	na	na	na	na	na	na

7.2.4 Length at start feeding

Provided as individual lengths (cm).

		Individual No									
Tank No	Treatment	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
1	C2	2.395	2.356	2.536	2.459	2.483	2.385	2.236	2.350	2.613	2.324
2	E3	2.730	2.630	2.724	2.442	2.614	2.643	2.547	2.553	2.626	2.430
3	E1	2.370	2.463	2.461	2.250	2.433	2.171	2.223	2.132	2.198	2.366
4	E3	2.632	2.588	2.715	2.497	2.552	2.604	2.626	2.580	2.594	2.521
5	D1	2.406	2.529	2.220	2.350	2.473	2.330	2.457	2.244	2.338	2.332
6	C1	2.449	2.457	2.553	2.639	2.557	2.488	2.432	2.496	2.389	2.525
7	C2	2.614	2.567	2.577	2.390	2.655	2.700	2.481	2.639	2.598	2.634
8	B3	2.770	2.542	2.713	2.491	2.480	2.401	2.591	2.439	2.682	2.497
9	F2	2.412	2.390	2.330	2.376	2.371	2.315	2.295	2.404	2.157	2.346
10	B2	2.484	2.540	2.454	2.678	2.428	2.443	2.539	2.406	2.431	2.414
11	F3	2.534	2.428	2.547	2.555	2.538	2.436	2.462	2.487	2.534	2.571
12	E1	2.445	2.410	2.502	2.277	2.405	2.271	2.092	2.315	2.455	2.429
13	B2	2.512	2.617	2.643	2.566	2.477	2.637	2.471	2.596	2.750	2.564
14	C3	2.452	2.636	2.633	2.707	2.510	2.505	2.624	2.470	2.265	2.697
15	F2	2.438	2.321	2.321	2.355	2.373	2.416	2.342	2.528	2.415	2.151
16	A2	2.617	2.569	2.474	2.525	2.609	2.741	2.573	2.494	2.574	2.606
17	B1	2.562	2.591	2.642	2.385	2.532	2.491	2.407	2.522	2.439	2.384
18	A3	2.549	2.473	2.575	2.497	2.454	2.363	2.345	2.438	2.533	2.469
19	F1	2.174	2.273	2.267	2.342	2.264	2.404	2.367	2.369	2.275	2.314
20	D2	2.564	2.604	2.537	2.673	2.380	2.566	2.497	2.578	2.622	2.603
21	A3	2.561	2.609	2.520	2.549	2.626	2.460	2.453	2.369	2.520	2.454
22	D2	2.437	2.492	2.732	2.628	2.521	2.339	2.414	2.676	2.651	2.625
23	D3	2.555	2.585	2.381	2.732	2.713	2.821	2.446	2.240	2.352	2.439
24	D3	2.670	2.766	2.807	2.627	2.790	2.726	2.640	2.647	2.761	2.540
25	A1	2.465	2.480	2.484	2.533	2.393	2.391	2.529	2.586	2.511	2.533
26	E2	2.366	2.179	2.448	2.339	2.175	2.370	2.343	2.337	2.361	2.164
27	A2	2.459	2.698	2.365	2.341	2.638	2.484	2.651	2.444	2.481	2.498
28	D1	2.220	2.545	2.335	2.620	2.428	2.542	2.404	2.492	2.578	2.222
29	A1	2.447	2.503	2.449	2.499	2.339	2.544	2.498	2.722	2.434	2.416
30	B1	2.601	2.553	2.631	2.595	2.612	2.681	2.628	2.516	2.464	2.509
31	C1	2.678	2.483	2.615	2.451	2.389	2.448	2.444	2.491	2.494	2.358
32	C3	2.537	2.574	2.531	2.514	2.674	2.738	2.525	2.389	2.598	2.658
33	F3	2.345	2.342	2.443	2.389	2.450	2.562	2.435	2.439	2.391	2.449
34	B3	2.607	2.520	2.526	2.369	2.639	2.529	2.753	2.780	2.605	2.521
35	E2	2.580	2.542	2.398	2.438	2.323	2.396	2.380	2.424	2.284	2.371
36	F1	2.317	2.362	2.418	2.270	2.318	2.321	2.214	2.337	2.183	2.258

7.2.5 Egg weight

Provided as individual weights (g wet weight).

Tank No	Treatment	Individual No. No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
1	C2	0.17	0.13	0.10	0.14	0.13	0.17	0.12	0.14	0.15	0.13
2	E3	0.14	0.15	0.12	0.15	0.10	0.20	0.10	0.13	0.11	0.13
3	E1	0.12	0.15	0.15	0.14	0.15	0.11	0.11	0.15	0.16	0.12
4	E3	0.12	0.16	0.16	0.14	0.12	0.14	0.13	0.13	0.14	0.16
5	D1	0.12	0.14	0.13	0.11	0.14	0.12	0.14	0.12	0.14	0.14
6	C1	0.17	0.17	0.16	0.13	0.12	0.17	0.13	0.13	0.11	0.10
7	C2	0.13	0.13	0.11	0.13	0.12	0.15	0.15	0.15	na	na
8	B3	0.16	0.13	0.14	0.13	0.13	0.16	0.13	0.14	0.12	0.10
9	F2	0.16	0.14	0.14	0.14	0.15	0.13	0.13	0.15	0.13	0.15
10	B2	0.13	0.12	0.13	0.10	0.17	0.13	0.12	0.11	0.11	0.12
11	F3	0.12	0.16	0.13	0.14	0.12	0.10	0.11	0.13	0.15	0.12
12	E1	0.17	0.15	0.17	0.15	0.12	0.13	0.17	0.10	0.11	0.13
13	B2	0.14	0.13	0.15	0.12	0.12	0.13	0.17	0.13	0.13	0.15
14	C3	0.15	0.15	0.12	0.14	0.13	0.14	0.15	0.13	0.13	0.13
15	F2	0.12	0.15	0.13	0.13	0.16	0.12	0.13	0.16	0.15	na
16	A2	0.13	0.13	0.13	0.12	0.13	0.12	0.13	0.13	0.13	0.15
17	B1	0.13	0.13	0.14	0.15	0.12	0.16	0.12	0.13	0.16	0.12
18	A3	0.12	0.13	0.12	0.15	0.15	0.14	0.12	0.14	0.14	0.14
19	F1	0.16	0.14	0.14	0.16	0.13	0.13	0.12	0.14	0.13	0.12
20	D2	0.13	0.14	0.12	0.15	0.14	0.13	0.12	0.13	0.11	0.14
21	A3	0.16	0.16	0.12	0.16	0.12	0.15	0.12	0.09	0.13	0.13
22	D2	0.13	0.13	0.14	0.13	0.12	0.14	0.14	0.13	0.14	0.15
23	D3	0.14	0.16	0.13	0.15	0.13	0.15	0.13	0.12	0.14	0.10
24	D3	0.13	0.14	0.16	na	na	0.15	0.15	0.13	0.12	0.12
25	A1	0.12	0.13	0.16	0.17	0.17	0.15	0.15	0.17	0.16	0.16
26	E2	0.14	0.18	0.13	0.12	0.12	0.16	0.16	0.15	0.14	0.14
27	A2	0.13	0.12	0.16	0.13	0.13	0.13	0.12	0.13	0.17	0.13
28	D1	0.13	0.14	0.12	0.13	0.12	0.12	0.16	0.13	0.13	0.16
29	A1	0.10	0.12	0.13	0.14	0.12	0.18	0.12	0.14	0.12	0.13
30	B1	0.13	0.13	0.14	0.12	0.13	0.14	0.16	0.13	0.11	0.14
31	C1	0.13	0.13	0.13	0.14	0.11	0.12	0.12	0.15	0.16	0.16
32	C3	0.12	0.11	0.15	0.12	0.10	0.16	0.12	0.13	na	na
33	F3	0.13	0.12	0.14	0.12	0.12	0.14	0.11	0.12	0.16	0.14
34	B3	0.12	0.11	0.11	0.12	0.10	0.15	0.14	0.15	0.13	0.15
35	E2	0.14	0.13	0.14	0.15	0.14	0.14	0.15	0.14	0.12	0.13
36	F1	0.14	0.11	0.12	0.14	0.17	0.15	0.14	0.13	0.12	0.16

7.2.6 Weight at eyeing

Provided as individual weights (g wet weight).

		Individual No.									
Tank No	Treatment	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
1	C2	0.15	0.11	0.15	0.13	0.15	0.13	0.15	0.13	0.11	0.14
2	E3	0.16	0.13	0.13	0.14	0.13	0.13	0.15	0.11	0.11	0.13
3	E1	0.17	0.14	0.17	0.14	0.15	0.15	0.16	0.14	0.16	0.16
4	E3	0.12	0.12	0.11	0.12	0.12	0.14	0.15	0.10	0.14	0.13
5	D1	0.17	0.13	0.13	0.11	0.17	0.15	0.15	0.13	0.15	0.13
6	C1	0.13	0.17	0.12	0.13	0.15	0.14	0.14	0.12	0.15	0.17
7	C2	0.13	0.13	0.14	0.16	0.15	0.16	0.14	0.13	0.14	0.13
8	B3	0.13	0.13	0.13	0.12	0.15	0.12	0.16	0.16	0.13	0.10
9	F2	0.13	0.13	0.16	0.13	0.11	0.12	0.13	0.16	0.17	0.13
10	B2	0.12	0.17	0.12	0.11	0.16	0.17	0.17	0.14	0.14	0.13
11	F3	0.13	0.13	0.11	0.13	0.13	0.16	0.12	0.16	0.12	0.15
12	E1	0.15	0.14	0.15	0.11	0.15	0.12	0.15	0.12	0.14	0.11
13	B2	0.12	0.13	0.15	0.16	0.14	0.16	0.17	0.14	0.14	0.15
14	C3	0.16	0.13	0.13	0.14	0.13	0.12	0.16	0.16	0.12	0.12
15	F2	0.17	0.12	0.14	0.12	0.14	0.13	0.12	0.12	0.13	0.15
16	A2	0.14	0.14	0.12	0.15	0.14	0.15	0.17	0.14	0.13	0.13
17	B1	0.14	0.15	0.13	0.14	0.15	0.12	0.17	0.15	0.14	0.15
18	A3	0.11	0.10	0.10	0.15	0.14	0.12	0.14	0.13	0.12	0.13
19	F1	0.15	0.15	0.12	0.10	0.13	0.13	0.12	0.17	0.11	0.15
20	D2	0.14	0.13	0.14	0.13	0.14	0.13	0.15	0.13	0.16	0.11
21	A3	0.15	0.13	0.11	0.13	0.12	0.16	0.16	0.15	0.11	0.12
22	D2	0.12	0.13	0.16	0.13	0.11	0.12	0.12	0.12	0.12	0.11
23	D3	0.14	0.14	0.11	0.12	0.14	0.13	0.13	0.14	0.13	0.13
24	D3	0.13	0.11	0.15	0.12	0.12	0.14	0.16	0.12	0.11	0.12
25	A1	0.14	0.13	0.14	0.14	0.14	0.13	0.13	0.14	0.14	0.12
26	E2	0.13	0.14	0.10	0.13	0.12	0.12	0.15	0.16	0.13	0.10
27	A2	0.15	0.16	0.11	0.16	0.14	0.15	0.17	0.13	0.12	0.13
28	D1	0.14	0.14	0.16	0.11	0.11	0.16	0.15	0.15	0.14	0.13
29	A1	0.12	0.14	0.15	0.13	0.13	0.14	0.11	0.14	0.13	0.10
30	B1	0.17	0.12	0.17	0.13	0.15	0.12	0.13	0.13	0.14	0.12
31	C1	0.15	0.15	0.13	0.11	0.13	0.14	0.13	0.12	0.15	0.17
32	C3	0.13	0.13	0.11	0.12	0.14	0.13	0.13	0.13	0.12	0.15
33	F3	0.16	0.12	0.14	0.14	0.12	0.12	0.13	0.12	0.13	0.16
34	B3	0.15	0.13	0.15	0.14	0.11	0.17	0.12	0.14	0.13	0.11
35	E2	0.13	0.12	0.12	0.14	0.14	0.15	0.13	0.13	0.12	0.12
36	F1	0.16	0.12	0.15	0.14	0.13	0.13	0.13	0.14	0.14	0.12

7.2.7 Weight at hatch

Provided as individual weights (mg wet weight).

Tank No	Treatment	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
		Individual No.									
1	C2	na	na	na	na	na	na	na	na	na	na
2	E3	15	11	10	11	12	16	10	12	11	10
3	E1	11	11	11	13	12	13	13	10	13	12
4	E3	12	11	13	12	14	15	13	11	11	15
5	D1	11	13	14	13	11	14	10	11	12	15
6	C1	12	14	13	9	19	9	13	13	15	13
7	C2	13	14	13	11	13	13	13	12	13	14
8	B3	12	13	10	10	15	13	13	11	11	14
9	F2	12	12	12	11	10	16	12	14	14	16
10	B2	17	12	11	11	12	12	11	11	14	11
11	F3	10	11	13	13	16	13	12	12	10	15
12	E1	11	11	14	14	11	13	11	11	10	10
13	B2	15	9	14	12	12	13	10	13	12	13
14	C3	10	15	12	16	12	11	12	14	16	15
15	F2	13	12	10	12	9	14	12	13	13	11
16	A2	12	10	11	14	14	14	11	11	14	15
17	B1	15	14	11	12	10	14	11	15	13	11
18	A3	15	12	15	13	12	11	11	11	15	11
19	F1	13	13	9	11	12	12	15	11	14	11
20	D2	11	14	17	11	12	14	11	13	12	16
21	A3	14	12	15	13	14	11	14	14	16	16
22	D2	17	16	12	12	11	14	12	7	18	12
23	D3	12	16	7	16	14	12	11	15	13	12
24	D3	13	11	13	10	14	14	12	11	16	12
25	A1	12	16	12	16	16	14	11	13	12	12
26	E2	11	12	14	14	12	10	14	13	10	13
27	A2	13	12	16	12	11	13	11	14	14	12
28	D1	13	10	10	14	12	9	12	14	12	14
29	A1	12	13	13	14	16	10	9	14	13	12
30	B1	12	11	14	10	13	12	13	13	12	13
31	C1	10	12	12	13	15	16	12	13	15	13
32	C3	12	12	15	14	11	14	13	11	14	14
33	F3	18	15	9	14	14	12	14	14	15	10
34	B3	14	13	15	9	13	13	12	13	11	13
35	E2	11	13	10	12	12	15	14	14	11	11
36	F1	na	na	na	na	na	na	na	na	na	na

7.2.8 Weight at start feeding

Provided as individual weights (g wet weight).

		Individual No.									
Tank No	Treatment	No 1	No 2	No 3	No 4	No 5	No 6	No 7	No 8	No 9	No 10
1	C2	0.16	0.13	0.14	0.13	0.12	0.14	0.12	0.12	0.12	0.14
2	E3	0.10	0.12	0.09	0.14	0.12	0.12	0.16	0.14	0.14	0.11
3	E1	0.10	0.14	0.15	0.12	0.11	0.12	0.10	0.13	0.10	0.13
4	E3	0.11	0.17	0.14	0.12	0.13	0.16	0.14	0.12	0.16	0.11
5	D1	0.12	0.14	0.10	0.11	0.16	0.13	0.15	0.10	0.11	0.12
6	C1	0.17	0.16	0.13	0.14	0.16	0.14	0.14	0.10	0.13	0.13
7	C2	0.12	0.13	0.13	0.12	0.15	0.16	0.11	0.17	0.17	0.17
8	B3	0.12	0.12	0.13	0.16	0.14	0.14	0.12	0.13	0.17	0.14
9	F2	0.16	0.14	0.13	0.12	0.11	0.16	0.10	0.11	0.15	0.13
10	B2	0.10	0.11	0.14	0.13	0.12	0.11	0.16	0.11	0.14	0.12
11	F3	0.10	0.13	0.12	0.12	0.15	0.10	0.13	0.13	0.12	0.15
12	E1	0.15	0.14	0.16	0.11	0.14	0.11	0.09	0.10	0.15	0.12
13	B2	0.14	0.16	0.17	0.13	0.12	0.14	0.10	0.16	0.18	0.14
14	C3	0.12	0.17	0.17	0.16	0.14	0.14	0.15	0.14	0.10	0.16
15	F2	0.14	0.12	0.11	0.12	0.10	0.13	0.13	0.14	0.12	0.08
16	A2	0.17	0.14	0.16	0.14	0.13	0.17	0.14	0.11	0.15	0.14
17	B1	0.16	0.15	0.18	0.12	0.15	0.14	0.14	0.15	0.12	0.15
18	A3	0.16	0.14	0.17	0.14	0.16	0.14	0.14	0.12	0.15	0.13
19	F1	0.11	0.11	0.11	0.12	0.10	0.12	0.11	0.11	0.10	0.12
20	D2	0.14	0.12	0.16	0.17	0.12	0.16	0.11	0.14	0.14	0.15
21	A3	0.14	0.17	0.17	0.16	0.16	0.15	0.12	0.10	0.15	0.13
22	D2	0.12	0.13	0.17	0.15	0.13	0.12	0.12	0.14	0.14	0.15
23	D3	0.14	0.15	0.12	0.15	0.15	0.19	0.13	0.11	0.09	0.13
24	D3	0.15	0.15	0.22	0.15	0.19	0.15	0.15	0.13	0.15	0.13
25	A1	0.14	0.15	0.14	0.13	0.12	0.15	0.15	0.17	0.16	0.14
26	E2	0.11	0.11	0.13	0.12	0.09	0.12	0.12	0.11	0.11	0.10
27	A2	0.11	0.17	0.11	0.10	0.19	0.13	0.17	0.14	0.12	0.13
28	D1	0.08	0.15	0.10	0.15	0.13	0.13	0.13	0.14	0.13	0.09
29	A1	0.15	0.16	0.13	0.14	0.12	0.17	0.16	0.17	0.13	0.15
30	B1	0.14	0.13	0.12	0.12	0.16	0.17	0.15	0.16	0.13	0.13
31	C1	0.16	0.17	0.15	0.13	0.12	0.15	0.12	0.14	0.10	0.12
32	C3	0.16	0.16	0.15	0.13	0.17	0.17	0.16	0.14	0.16	0.16
33	F3	0.11	0.15	0.13	0.13	0.12	0.10	0.13	0.12	0.10	0.14
34	B3	0.16	0.11	0.14	0.16	0.14	0.15	0.14	0.19	0.15	0.18
35	E2	0.14	0.11	0.13	0.12	0.15	0.14	0.13	0.15	0.12	0.14
36	F1	0.11	0.10	0.15	0.13	0.13	0.13	0.11	0.16	0.10	0.14

7.2.9 Metal concentrations in body tissue

Provided as pooled concentrations of 10 individuals per replicate at µg/g wet weight of body tissue.

		Concentrations (µg/g wet weight)					
		Egg		Eyeing		Start feed	
Tank No	Treatment	Cd	Ca	Cd	Ca	Cd	Ca
1	C2	0.015	700	0.023	733	0.010	718
2	E3	0.059	761	0.101	727	0.041	734
3	E1	0.343	692	0.471	734	0.049	653
4	E3	0.061	770	0.093	760	0.050	778
5	D1	0.105	708	0.118	699	0.054	628
6	C1	0.042	668	0.042	705	0.072	773
7	C2	0.019	732	0.026	679	0.019	786
8	B3	0.005	730	0.005	722	0.006	798
9	F2	0.397	741	0.428	692	0.103	670
10	B2	0.008	728	0.009	716	0.017	768
11	F3	0.170	780	0.263	744	0.104	667
12	E1	0.337	685	0.317	679	0.074	627
13	B2	0.008	729	0.009	719	0.020	818
14	C3	0.009	737	0.013	774	0.009	904
15	F2	0.369	765	0.430	743	0.112	647
16	A2	0.003	706	0.003	686	0.003	762
17	B1	0.014	719	0.014	662	0.075	751
18	A3	0.003	769	0.003	707	0.004	699
19	F1	0.937	700	0.944	692	0.148	647
20	D2	0.048	700	0.052	737	0.063	896
21	A3	0.003	748	0.003	701	0.002	709
22	D2	0.044	717	0.041	717	0.060	842
23	D3	0.019	747	0.026	729	0.026	901
24	D3	0.023	734	0.034	729	0.027	875
25	A1	0.004	742	0.004	690	0.007	773
26	E2	0.129	705	0.198	701	0.092	679
27	A2	0.004	710	0.003	693	0.003	648
28	D1	0.098	699	0.114	692	0.076	665
29	A1	0.004	683	0.003	672	0.008	905
30	B1	0.017	688	0.017	629	0.057	733
31	C1	0.049	679	0.048	683	0.088	767
32	C3	0.008	726	0.014	753	0.009	830
33	F3	0.173	747	0.210	738	0.098	636
34	B3	0.005	767	0.005	746	0.005	854
35	E2	0.127	699	0.173	734	0.059	692
36	F1	0.990	731	0.964	678	0.088	654